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Vancomycin-loaded nanobubbles: A new platform for controlled antibiotic delivery against methicillin-resistant *Staphylococcus aureus* infections

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Abstract: Vancomycin (Vm) currently represents the gold standard against methicillin-resistant *Staphylococcus aureus* (MRSA) infections. However, it is associated with low oral bioavailability, formulation stability issues, and severe side effects upon systemic administration. These drawbacks could be overcome by Vm topical administration if properly encapsulated in a nanocarrier. Intriguingly, nanobubbles (NBs) are responsive to physical external stimuli such as ultrasound (US), promoting drug delivery. In this work, perfluoropentane (PFP)-cored NBs were loaded with Vm by coupling to the outer dextran sulfate shell. Vm-loaded NBs (VmLNBS) displayed ~300 nm sizes, anionic surfaces and good drug encapsulation efficiency. In vitro, VmLNBS showed prolonged drug release kinetics, not accompanied by cytotoxicity on human keratinocytes. Interestingly, VmLNBS were generally more effective than Vm alone in MRSA killing, with VmLNB antibacterial activity being more sustained over time as a result of prolonged drug release profile. Besides, VmLNBS were not internalized by staphylococci, opposite to Vm solution. Further US association promoted drug delivery from VmLNBS through an in vitro model of porcine skin. Taken together, these results support the hypothesis that proper Vm encapsulation in US-responsive NBs might be a promising strategy for the topical treatment of MRSA wound infections.

Torino, Italy: 14th March 2017

To the Editor
of the International Journal of Pharmaceutics

Dear Editor,

please find attached here the revised version (both marked and clean copies) of our research article titled "Vancomycin-loaded nanobubbles: a new platform for controlled antibiotic delivery against methicillin-resistant *Staphylococcus aureus* infections".

As requested, the manuscript was implemented according to the reviewer's suggestions and all the references were modified according to the journal's author guidelines. Following the reviewer's comments, the image quality was improved for all figures. A rebuttal letter containing our replies to the author(s)'s comments is also attached.

We sincerely hope that you will find the revised version of the manuscript acceptable for publication by the International Journal of Pharmaceutics.

We are looking forward to receiving your feedback.

Kind regards

Prof. Roberta Cavalli

IJP AUTHOR CHECKLIST

Dear Author,

It frequently happens that on receipt of an article for publication, we find that certain elements of the manuscript, or related information, is missing. This is regrettable of course since it means there will be a delay in processing the article while we obtain the missing details.

In order to avoid such delays in the publication of your article, if accepted, could you please run through the list of items below and make sure you have completed the items.

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General

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Reviewers' comments:

Reviewer #1: Comments:

1. The quality of figures should be greatly improved, especially the graphical abstract, Figure 3 and 6.

We apologize for such an issue. According to the reviewer's suggestion, the image quality was improved for all figures.

2. In line 159, the section 2.2.1., How many ml of dextran sulfate aqueous solutions were added into the Vm solution? What is the solvent of Vm solution? Water or other organic solvents? When centrifuge the unbound Vm, how much is the centrifugal speed? And the centrifugal time also should be provided.

One ml of dextran sulfate aqueous solution at increasing concentrations was added into 1 ml of vancomycin aqueous solution. The centrifugal speed used was 20000 rpm for 15 minutes. The manuscript was implemented with such information (Materials&Methods section, par. 2.2.1).

3. For preparing pre-emulsion containing Epikuron® 200, palmitic acid and PFP, how much g/mg of PFP was used? To prepare polymeric NBs, How many ml of dextran sulfate aqueous solution was added into the PFP emulsion?

The amount of PFP and dextran sulfate used for each nanobubble formulation were 500 µL and 350 µL, respectively. Such information was added in the text (Materials&Methods section, par. 2.2.2).

4. In line 199, for the TEM observation, the type and brand for the TEM equipment should be provided in the text.

The type and brand of the instrument used for TEM analyses (Philips CM10 (Eindhoven, NL)) were added in the text (Materials&Methods section, par. 2.3.1).

5. To measure the loading capacity, the VmLNBS solution was sonicated and centrifuged, and then the supernatant was analyzed. How to validate the VmLNBS were completely broken? Why not use the organic solvent to destroy the structure of the VmLNBS?

The parameters of the used freeze-drying process are severe to maintain the integrity of the nanostructure in the absence of any cryoprotectors. Preliminary experiments were carried out to evaluate by optical microscopy the nanobubble structure and to set a protocol suitable for determining the loading capacity. The manuscript was modified accordingly (Materials&Methods section, par. 2.3.5).

6. In line 285, the unit of centrifugal speed was g, in line 230, the unit is rpm. The author should check them. Some similar expressions also should be uniformed, such as mL and microL.

All units of centrifugal speed as well as those indicating microliters were uniformed throughout the full text.

7. The viscosity of VmLNBs was higher than that of NB. The reason should be explained in the section of "Discussion". Does the change of viscosity affect loading capacity, encapsulation efficiency, physical stability, Vm release, and permeation efficiency?

We apologize for the typing mistake concerning the viscosity value of blank NB formulations. We determined again the viscosity using the Ubbelohde capillary viscosimeter to confirm the data. The viscosity of all NB formulations (i.e. blank NBs, VmLNBs, fluorescent NBs, and fluorescent VmLNBs) did not show any significant changes. A specific sentence was added in the text (Results section par. 3.1).

8. Table 2 can be incorporated in Table 1.

According to the reviewer's suggestion, Table 2 was incorporated in Table1.

Reviewer #2: The development of novel systems for antibiotics is in its infancy as compared to other disease conditions and is receiving increasing interest in the literature. Whilst several nanosystems are being reported for vancomycin, few, if any have been with nanobubbles. Further, transdermal delivery of nanoencapsulated antibiotics is an emerging research area. This paper describes the encapsulation of vancomycin into nanobubbles for ultrasound mediated drug release and also to bypass the stratum corneum to optimize the treatment of wound infections. This proof of concept study is well designed and the potential of this delivery system is demonstrated. The paper is well written with some minor recommendations:

1. Images of the nanobubbles show one with a single nanobubble and another with 2. Ideally an image showing a population representation should be considered.

A TEM image showing a population representation of VmLNBs was added in the Supplementary Information.

2. There are several inconsistencies in the referencing style which need to be corrected.

All the references were modified according to the journal's author guidelines.

3. Pg 18, Line 368. The last sentence is incorrect and needs to be rewritten.

According to the reviewer's suggestion, we changed the sentence as follows: "As shown in Figure 3, the drug resulted much more stable from a chemical point of view when properly incorporated in the nanocarrier (VmLNBs) than as such in solution." (Results section, par. 3.2)

Reviewer #3: Manuscript IJP-D-16-02774 "Vancomycin - loaded nanobubbles: a new platform for controlled..." by Argenziano et al. describes the fabrication, characterization and release capability of polymer shelled droplets loaded with vancomycin.

The manuscript should be implemented according to the following comments:

1) It should be specified whether PFP is liquid.

Perfluoropentane is a perfluorocarbon with a boiling point of 29°C, hence liquid at room temperature. The use of PFP allows liquid droplet generation at room temperature. Then, PFP in nanodroplets can be activated by an external stimulus, like ultrasound, by means of a mechanism called acoustic droplet vaporization, causing the droplet to become a bubble. The sentence was added in the manuscript (Introduction section, lines 117-120).

2) Term "nanobubbles" can be misleading. At room temperature the core of the particles is liquid PFP. Therefore nanobubbles does not describes correctly such particles. It would be more proper the term "nanodroplets" or "nanovesicles"

The formulation is referred to as "nanobubbles" for sake of simplicity (to distinguish them from so-called decafluoropentane-containing nanodroplets, already patented by our group; see Introduction section for more details about those nanodroplets) but we acknowledge that, prior to the application of ultrasound, it would be more precise to use the term "nanodroplets" when the core is constituted of perfluoropentane. This clarification was included in the text (Introduction section, lines 129-132).

3) Figures are not numbered and are very low in resolution (including the graphical abstract). Sometimes they are not readable.

We apologize for the low quality of figures. According to the reviewer's suggestion, the resolution of all figures was improved. Also, Figures were numbered in accordance to legend numbers.

4) Scheme of the particle differs from the particle description of the graphical abstract in the position of vancomycin, tethered to the external surface of the particle and in the particle shell, respectively.

Vancomycin is included in the polysaccharide shell. For clarity, we modified Figure 1.

5) Viscosity measurements obtained by capillary viscosimetry should be defined. With an Ubbelohde capillary viscometer a relative viscosity, a specific viscosity, an intrinsic viscosity can be obtained. Which one is reported ? All of them have different dimensions from the reported one, i.e. cP. Relative (to solvent) and specific viscosities are dimensionless, intrinsic viscosity has dimension of an inverse of concentration.

With the Ubbelohde capillary viscometer, the time required for the nanosuspension to flow through a capillary of a known diameter of a certain factor (K) between two marked points was measured. By multiplying the time taken, by the factor of the viscometer (0.105), the kinematic viscosity was obtained. The dynamic viscosity was obtained by multiplying kinematic viscosity by density. The cP is the unit of dynamic viscosity in the metric CGS (centimeter-gram-second) system.

6) Vancomycin permeation study puzzled me a lot. The experiment should be conducted at osmotic conditions. To avoid Donnan effects with a charged not diffusible solute, i.e. nanobubbles, a suitable diffusible ionic strength should be used on both the compartments separated by the membrane. According to the given description NaCl 0.9 % w/w has been added only on one compartment. In this conditions other, not controlled contributions affects the diffusion process of vancomycin. In the description of the set up, the concentration of nanobubbles is not reported.

We apologize for the inaccurate description of the experimental setup. For *in vitro* permeation studies, NB samples were prepared in saline solution (NaCl 0.9% w/v). The concentration of NBs in the donor phase was 1×10^{12} NBs/ml. All information was added to the text (Materials&Methods section, par. 2.2.2 and 2.5).

7) When ultrasound are applied, it is important to check the behaviour of the "nanobubbles" (nanodroplets) in order to frame the enhanced release. Do "nanobubbles" (nanodroplets) undergo acoustic droplet vaporization? This effect is known to transform droplets into bubbles, thus changing the release of the payload.

Nanobubbles were observed by US standard imaging (MyLab ESAOTE instrument) and they showed a good scattering response, either in the absence or presence of vancomycin. Further investigations are needed to check whether actual vaporization occurred. With regard to the drug release, preliminary experiments showed an enhanced release kinetics after US application.

8) Pg 6 line 115: PFP is liquid or gas ?

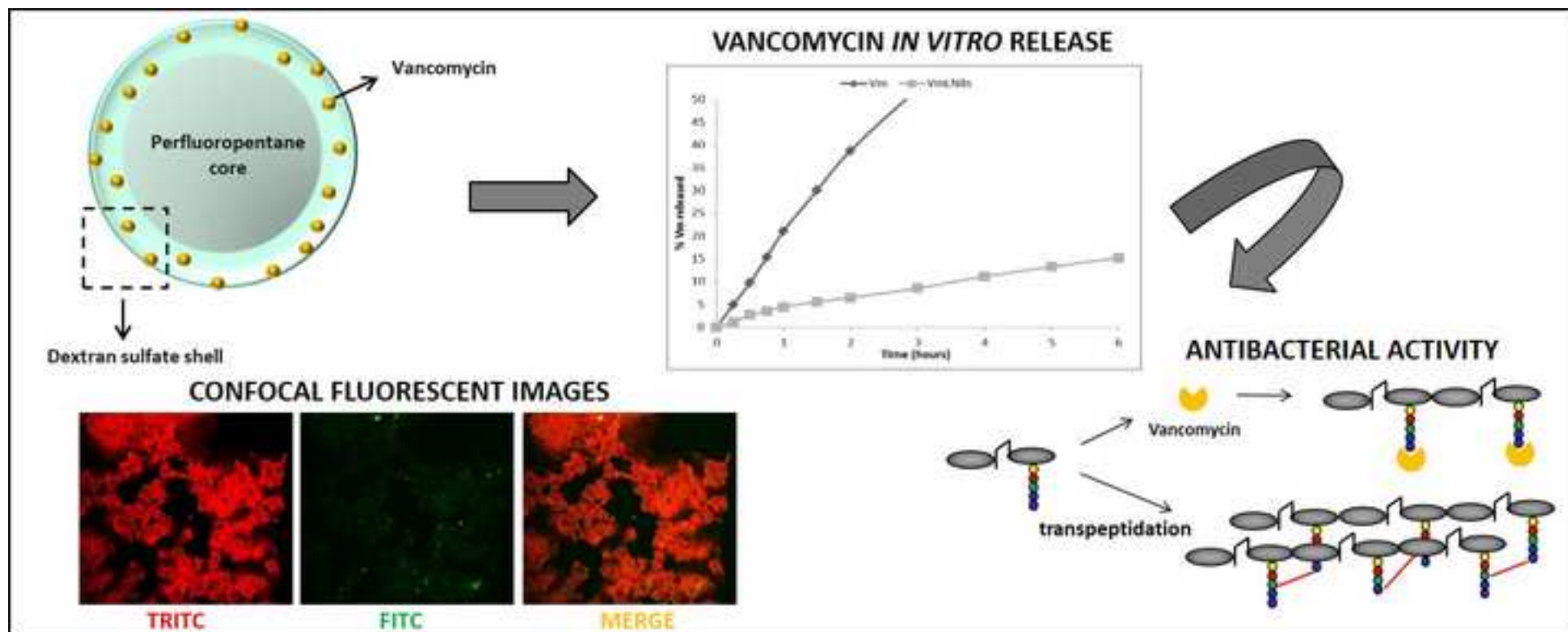
Perfluoropentane is liquid at room temperature, having a boiling point of about 29 °C. Therefore, it is gaseous at body temperature (37°C) as such.

9) Pg 15 line 332: why in confocal microscopy imaging, bacteria are dried ?

The drying of bacteria is a step necessary for their staining. After smearing of bacteria on the glass-slide, every staining procedure considers that bacteria have to be air-dried to fix them on the slide and to avoid the subsequent rinsing of the smear during staining procedure, as well as to allow the sample to more readily take up stain(s).

10) Vancomycin hydrochloride is not mentioned in the Material section.

Vancomycin hydrochloride was from Sigma-Aldrich (St Louis, MO). Therefore, it falls into the general sentence "All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows" (first sentence of par. 2.1 in Materials&Methods section).



Vancomycin-loaded nanobubbles: a new platform for controlled antibiotic delivery against methicillin-resistant *Staphylococcus aureus* infections.

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Abstract

Vancomycin (Vm) currently represents the gold standard against methicillin-resistant *Staphylococcus aureus* (MRSA) infections. However, it is associated with low oral bioavailability, formulation stability issues, and severe side effects upon systemic administration. These drawbacks could be overcome by Vm topical administration if properly encapsulated in a nanocarrier. Intriguingly, nanobubbles (NBs) are responsive to physical external stimuli such as ultrasound (US), promoting drug delivery. In this work, perfluoropentane (PFP)-cored NBs were loaded with Vm by coupling to the outer dextran sulfate shell. Vm-loaded NBs (VmLNBs) displayed ~300 nm sizes, anionic surfaces and good drug encapsulation efficiency. *In vitro*, VmLNBs showed prolonged drug release kinetics, not accompanied by cytotoxicity on human keratinocytes. Interestingly, VmLNBs were generally more effective than Vm alone in MRSA killing, with VmLNB antibacterial activity being more sustained over time as a result of prolonged drug release profile. Besides, VmLNBs were not internalized by *staphylococci*, opposite to Vm solution. Further US association promoted drug delivery from VmLNBs through an *in vitro* model of porcine skin. Taken together, these results support the hypothesis that proper Vm encapsulation in US-responsive NBs might be a promising strategy for the topical treatment of MRSA wound infections.

Key words

Nanobubbles; vancomycin; methicillin-resistant *Staphylococcus aureus*; ultrasound; prolonged release.

47 **1. Introduction**

48

49 Chronic wounds fail to proceed through timely regulated and interrelated processes to restore
50 anatomical and functional integrity of the injured tissues (Lazarus et al., 1994) such as diabetic feet,
51 bedsores, and venous ulcers (Markova et al., 2012). To date, these types of wounds are considered like
52 a silent epidemic, affecting a large fraction of the world population and posing a major gathering threat
53 to the public health and economy of all developed countries (Daeschlein, 2013). Hospitalized patients
54 are at particular risk, especially those suffering from diabetes, human immunodeficiency virus or other
55 immune disorders, as well as those undergoing chemotherapy (Payne et al., 2008).

56 Beyond delayed healing processes due to different factors (hypoxia, persistent inflammation, and
57 altered balances between tissue remodelling proteinases and their inhibitors), chronic wounds are often
58 worsened by microbial infections (Gurusamy et al., 2013). Among the bacteria responsible for skin
59 infection, *Staphylococcus aureus* represents the most common pathogen to be identified in chronic
60 wounds, with methicillin-resistant *S. aureus* (MRSA) accounting for upward of 20% to 50% of cases
61 (Price, 2010). MRSA colonies often develop at the interface between synthetic prostheses and
62 biological tissues, particularly during surgery and post-surgery course. In addition, MRSA colonization
63 or infection of wounds can result in MRSA bacteremia, which is associated with a 30-day mortality of
64 about 28% to 38% patients (Gurusamy et al., 2013).

65 The main goal of chronic wound treatment is to decrease the injuring-associated microbial load, thus
66 allowing wound healing processes to take place. However, conventional systemic delivery of
67 antibiotics not only entails poor penetration into ischemic and necrotic tissues, but can also cause
68 systemic toxicity with associated renal and liver complications, resulting in forced hospitalization for
69 further monitoring and advanced treatment. On the contrary, topically applied antimicrobials have
70 proven effective in decreasing bacterial levels in granulating wounds (Diehr et al., 2008). Therefore,

71 alternative local delivery of antimicrobials - either by topical administration or through novel delivery
72 devices - may enable to keep high local antibiotic concentrations for prolonged release times without
73 reaching systemic toxicity (Zilberman et al., 2008).

74 A promising approach to develop a topical therapy for microbial infection in skin and soft tissues
75 would employ biocompatible nanomaterials and drug nanocarriers. Indeed, nanotechnology represents
76 an emerging field to be exploited for antibiotic drug delivery. Thanks to their physical and chemical
77 properties (small size, high surface-to-volume ratio and suitable surface modification) nano-sized
78 materials may be used as drug carriers to trespass several physiological barriers and to reach biological
79 targets. The coupling of nanocarriers with anti-infectious agents makes it likely to increase drug
80 concentrations and drug penetration at the site of infection. As a result, it might not only improve the
81 therapeutic index but also reduce some issues associated with nonspecific cytotoxicity and antibiotic
82 resistance (Sharma et al., 2012).

83 Vancomycin hydrochloride, being effective against many Gram-positive bacteria that are unresponsive
84 to common antibiotics, represents the gold standard against MRSA infections (Kullar et al., 2016).
85 However, Vm is poorly absorbed from the gastrointestinal tract with a low oral bioavailability. Low
86 intravenous infusion is often suggested as a feasible alternative for drug administration, but Vm
87 instability in aqueous solutions at 37°C could imply a tremendous reduction of drug effectiveness
88 (Mawhinney et al., 1992; Raverdy et al., 2013). Following parenteral administration, Vm displays a
89 slow mode of action, a complex concentration-time profile, and a disappointingly low penetration in
90 tissues (Vandecasteele et al., 2012). Furthermore, systemic Vm administration can be associated with
91 several adverse effects (Vidal et al., 1992). On the other hand, Vm topical application – that would be
92 much safer than systemic administration - is currently limited by several factors such as skin barrier
93 properties and poor drug permeability (Giandalia et al., 2001). Being the main goal of chronic wound
94 treatment to decrease the microbial load, allowing the healing processes to take place, new delivery

95 protocol should be devised, since conventional systemic delivery of antibiotics requires a drug
96 concentration which is locally ineffective because of the poor penetration into ischemic and necrotic
97 tissues, but can cause systemic toxicity and topically applied antimicrobials have proven effective in
98 decreasing bacterial levels in granulating wounds (Diehr et al., 2007), without inducing systemic
99 toxicity (Zilberman et al., 2008) but suffer from poor diffusion across membranes.

100 Intriguingly, the use of a nanocarrier may help to avoid the abovementioned drawbacks. Notably,
101 nanocarriers such as liposomes, microemulsions, and lipid nanoparticles have the potential to deliver
102 drugs to the skin more efficiently than conventional topical carriers such as creams and ointments, that
103 are not usually recommended for applications on injured skin (Giandalia et al., 2001; Prabhu et al.,
104 2012). However, the response to drug topical applications has been too weak so far, mainly due to the
105 inability to cross the external skin barrier (*stratum corneum*) and reach the dermal regions where the
106 bacteria are nested. Interestingly, physical media such as ultrasound (US) are reportedly able to trigger
107 drug release at the site of infection by temporarily increasing skin permeability through sonophoresis.
108 As such, US is useful to promote drug targeting and transdermal delivery in a non-invasive manner
109 (Azagury et al., 2014; Park et al., 2012).

110 Microbubbles (MBs) (Guiot et al., 2006), nanobubbles (NBs) (Cavalli et al., 2009a; Cavalli et al.,
111 2009b; Cavalli et al., 2016) and nanodroplets (NDs) (Magnetto et al., 2014; Prato et al., 2015) are
112 suitable carriers to be combined with such a physical trigger. They are spherical core-shell structures
113 filled with gases such as perfluorocarbons. Particularly, oxygen-cored nanostructures can be employed
114 both for sonography (as contrast agents) (Fokong et al., 2012; Marxer et al., 2011) and for therapy (as
115 hypoxia- and infection-counteracting devices) (Gulino et al., 2015; Banche et al., 2015; Khadjavi et al.,
116 2015; Basilico et al., 2015; Prato et al., 2016). In particular NBs, consisting in an outer shell of a
117 biocompatible/biodegradable polysaccharide (chitosan, dextran, or dextran sulfate) and an inner core
118 filled with an oxygen-storing fluorocarbon (perfluoropentane, PFP), have been purposely developed as

119 a new non-invasive, low-cost and multipurpose nanotechnological platform (Cavalli et al., 2009a;
120 Cavalli et al., 2009b; Cavalli et al., 2016). PFP is a perfluorocarbon with a boiling point of 29°C, hence
121 liquid at room temperature. The use of PFP allows liquid droplet generation at room temperature. Then,
122 PFP in nanodroplets can be activated by an external stimulus, like US, by means of a mechanism called
123 acoustic droplet vaporization, causing the droplet to become a bubble. Depending on the properties of
124 the nanostructure, NBs can be subsequently coupled with different molecules, such as drugs or genetic
125 materials, thus acting as nanocarriers (Cavalli et al., 2012; Cavalli et al., 2013; Delalande et al., 2012;
126 Yin et al., 2014). Due to their structure and their gaseous core, NBs are very responsive to US and can
127 take advantage from a number of effects related to microcavitation and microstreaming, occurring at
128 the liquid-membrane interface and responsible for transitory and reversible openings of the pores, thus
129 crossing the membrane itself and delivering their content beyond the tissue (sonophoresis) or the cell
130 (sonoporation) membrane (Karshafian et al., 2009).

131 Based on these preconditions, the present work aimed at producing dextran sulfate-shelled and PFP-
132 cored NBs for Vm local delivery to potentially treat skin infectious diseases. The formulation is
133 referred to as “nanobubbles” for sake of simplicity but it must be said that, prior to the application of
134 US, it would be more accurate to use the term “nanodroplets” when the core is constituted of PFP.
135 Therefore, Vm-loaded NBs (VmLNBs) were prepared and characterized for physico-chemical
136 parameters and drug release kinetics; tested for biocompatibility with human skin cells and for their
137 antibacterial properties or interactions with MRSA; and challenged for responsiveness to US, in order
138 to assess their effectiveness as Vm nanocarriers for local delivery.

139

140 **2. Material and methods**

141

142 **2.1. Materials**

143 All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows. Sterile
144 plastics were from Costar, Cambridge, UK; ethanol (96%) was from Carlo Erba (Milan, Italy); soybean
145 lecithin (Epikuron 200[®]) was from Cargill (Hamburg, Germany); 1-800 Millipore system to obtain
146 ultrapure water and Amicon[®] Ultra-0.5 centrifugal filter device were from Millipore (Molsheim,
147 France); Ultra-Turrax SG215 homogenizer was from IKA (Staufen, Germany); RPMI 1640 medium
148 was from Invitrogen (Carlsbad, CA); Nanobrook 90Plus Particle Size Analyzer was from Brookhaven
149 (New York City, NY); Philips CM10 electron microscope was from Philips (Eindhoven, the
150 Netherlands); Ubbelohde capillary viscosimeter was from SCHOTT Instruments GmbH (Mainz,
151 Germany); Perkin Elmer PUMP 250B was from Perkin Elmer (Waltham, MA); Flexar UV/Vis LC
152 spectrophotometer detector was from Perkin Elmer (Waltham, MA); Agilent TC C₁₈ columns were
153 from Agilent (Santa Clara, CA); Orion Model 420A pH Meter was from Thermo Scientific (Waltham,
154 MA); Semi-Micro Osmometer K-7400 was from Knauer (Berlin, Germany); Beckman Coulter Allegra
155 64R Centrifuge was from Beckman Coulter (Brea, CA); Spectra/Por cellulose membranes were from
156 Spectrum Laboratories (Rancho Dominguez, CA); HaCaT cells were from Cell Line Service GmbH
157 (Eppelheim, Germany); cell culture RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM)
158 were from Invitrogen (Carlsbad, CA); streptomycin was from Cambrex Bio Science (Vervies,
159 Belgium); humidified CO₂/air-incubator was from Thermo Fisher Scientific Inc. (Waltham, MA);
160 tryptic soy broth (TSB) and tryptic soy agar (TSA) were from Merk KgaA (Darmstadt, Germany);
161 Olympus Fluoview 200 laser scanning confocal system mounted on an inverted IX70 Olympus
162 microscope was from Olympus America Inc. (Melville, NY, USA) ; SPSS 16.0 software was from
163 SPSS Inc. (Chicago, IL).

164 **2.2. Development and manufacturing of formulations**

165

166 **2.2.1. Determination of Vm and dextran sulfate interaction ratio**

167 Increasing concentrations (0.25, 0.5, 1.0, 2.0 mg/mL) of dextran sulfate aqueous solutions (1 mL) were
168 added to 1 mL of Vm aqueous solution (1 mg/mL) under magnetic stirring at room temperature
169 overnight. After equilibration, the systems were separated by centrifugation (20000 rpm, 15 minutes)
170 using a centrifugal filter device (Amicon[®] Ultra), in order to determine the amount of unbound Vm in
171 the filtrate phase. The drug concentration in the filtrate was determined using the HPLC method
172 described below.

173

174 **2.2.2. Preparation of NB, Vm, and VmLNB formulations**

175 NBs were formulated using PFP for the inner core and dextran sulfate for the shell. A purposely tuned
176 multi-step protocol was designed. Briefly, a pre-emulsion was obtained adding 300 µL of an ethanol
177 solution containing Epikuron[®] 200 and palmitic acid (1% w/v) to 500 µL of PFP under magnetic
178 stirring. After the addition of 4.8 mL of ultrapure water, the system was homogenized using a Ultra-
179 Turrax SG215 homogenizer. To obtain the polymeric NBs, 350 µL of 1% w/v dextran sulfate
180 (molecular weight = 100 kDa) aqueous solution was added drop-wise under magnetic stirring. Blank
181 NBs obtained according to this procedure were employed as control formulations in the subsequent
182 experiments. On the other hand, to obtain VmLNBs, an extra step based on drop-wise addition of a Vm
183 aqueous solution (pH 3.5) to the so-formed NBs was performed under mild stirring. Different
184 concentrations of Vm solutions were added to prepare a series of VmLNB formulations with increasing
185 drug content (0.004, 0.01, 0.1, and 1 mg/mL). VmLNBs were then purified by dialysis to eliminate
186 unbound molecules. For selected experiments, fluorescent NBs and VmLNBs were obtained by the

187 addition of 6-coumarin (1 mg/mL) to the PFP core. Alternatively, fluorescent Vm was synthesized
188 through reaction between fluorescein isothiocyanate (FITC) and Vm. For this purpose, an amount of
189 FITC solution in methanol (0.2 % w/v) was added to Vm aqueous solution and incubated under stirring
190 overnight in the dark. **Figure 1** shows a representative scheme resuming the general structure of
191 fluorescent VmLNBs. For cell experiments, NBs were prepared in phosphate buffer saline pH 7.4
192 (PBS). For *in vitro* permeation studies, NBs were prepared in saline solution (NaCl 0.9% w/v).

193

194 **2.2.3. NB sterilization**

195 Firstly, the glassware and the components were sterilized at 121 °C and 2 bar. Subsequently, all NB
196 formulations were sterilized through UV-C exposure for 20 min. Thereafter, UV-C-treated materials
197 were incubated with cell culture RPMI 1640 medium in a humidified CO₂/air-incubator at 37°C up to
198 72 h, not displaying any signs of microbial contamination when checked by optical microscopy.

199

200 **2.3. Characterization of formulations**

201

202 **2.3.1. Characterization of NB and VmLNB formulations**

203 The average diameter, polydispersity index and zeta potential were determined by photocorrelation
204 spectroscopy using a particle size analyzer at a scattering angle of 90° and a temperature of 25 °C. NB
205 suspensions were diluted in deionized filtered water before measurement. For zeta potential
206 determination, samples of diluted NB formulations were placed in the electrophoretic cell, where an
207 electric field of approximately 15 V/cm was applied. The morphology of formulations was evaluated
208 by Transmission Electron Microscopy (TEM), using a Philips CM10 (Eindhoven, NL) instrument. NB
209 and VmLNB aqueous suspensions were sprayed on Formwar-coated copper grid and air-dried before

210 observation. The viscosity of the samples was determined at 25 °C using a Ubbelohde capillary
211 viscosimeter.

212

213 ***2.3.2. HPLC quantitative Vm determination***

214 Vm quantitative determination was carried out by using an HPLC system based on a Perkin Elmer
215 pump equipped with a spectrophotometer detector. Analyses were performed using an Agilent TC C₁₈
216 column (250 mm × 4.6 mm, 5 µm). The mobile phase was a mixture of KH₂PO₄ 50 mM (pH 4) and
217 acetonitrile (92:8 v/v), degassed and pumped through the column with a flow rate of 1 mL/min.
218 Ultraviolet detection was set at 286 nm. The external standard method was used to calculate the drug
219 concentration. For this purpose, 1 mg of Vm was weighted, placed in a volumetric flask, and dissolved
220 in water to obtain a stock standard solution. This solution was then diluted using the mobile phase,
221 providing a series of calibration solutions, subsequently injected into the HPLC system. Linear
222 calibration curve was obtained over the concentration range of 0.5–25 µg/mL, with a regression
223 coefficient of 0.999.

224

225 ***2.3.3. In vitro evaluation of Vm stability***

226 Vm chemical stability - either solved in aqueous solution or loaded in VmLNBs - was evaluated at
227 room temperature and at 37 °C over time. A quantitative determination of Vm concentration over time
228 was carried out using the HPLC method described above.

229

230 ***2.3.4. NB stability over time and after US administration***

231 The physical stability of NBs was evaluated by morphological analysis and by size and zeta potential
232 determination of formulation over time. Their average diameters, zeta potential values and morphology
233 were assessed up to six months. Stability was also investigated following NB exposure to US ($f = 2.5 \pm$

234 0.1 MHz; t = 10 min; P = 5 W). NB morphology was observed by TEM to confirm the integrity of NB
235 structure.

236

237 **2.3.5. Encapsulation efficiency and loading capacity of Vm in NBs**

238 The encapsulation efficiency of VmLNBs was determined using a centrifugal filter system. 150 µL of
239 VmLNB suspension were put in an Amicon[®] Ultra-0.5 centrifugal filter device and centrifuged at
240 15000 rpm for 30 minutes using Beckman Coulter Allegra 64R Centrifuge. The solution filtered in the
241 bottom of the tube was quantified and after suitable dilution was analyzed by HPLC, in order to obtain
242 the concentration of free Vm in VmLNBs suspensions. The encapsulation efficiency was calculated by
243 subtracting the amount of free drug from the initial amount of added Vm, according to the following
244 equation:

$$245 \text{ Encapsulation efficiency} = \frac{(\text{total Vm} - \text{free Vm})}{\text{total Vm}} \times 100$$

246 The loading capacity was determined on freeze-dried NB samples. Briefly, a weighted amount of
247 freeze-dried VmLNBs was suspended in 10 mL of water. After sonication and centrifugation, the
248 supernatant was diluted with mobile phase and analyzed by HPLC. The loading capacity of Vm in
249 VmLNBs was calculated as follows:

$$250 \text{ Loading capacity} = \frac{(\text{total Vm} - \text{free Vm})}{\text{NB weight}} \times 100$$

251

252 **2.4. In vitro release studies**

253 *In vitro* drug release experiments were conducted in a multi-compartment rotating cell, comprising a
254 donor chamber separated by a cellulose membrane (cut-off = 12000 Da) from a receiving compartment.
255 One ml of VmLNB suspension at different concentrations (1, 0.1, 0.01 and 0.004 mg/mL) was placed

256 in the donor chamber. The *in vitro* release kinetics of Vm from VmLNB was compared to a Vm
257 aqueous solution (1 mg/mL) as a control. The receiving phase, containing phosphate buffer 0.05 M (pH
258 7.4) was withdrawn at regular intervals and replaced with the same amount of fresh buffer. Quantitative
259 determination of Vm in the withdrawn samples was carried out by the HPLC method, as described in
260 the previous paragraph. Data were expressed as % of Vm released over time.

261

262 **2.5. *In vitro* permeation study**

263 *In vitro* studies were performed using a vertical diffusion Franz cell to evaluate Vm permeation
264 throughout the skin. The Franz cell consists of a donor compartment, with Vm (1 mg/mL, either free or
265 carried by VmLNBs, 1×10^{12} NBs/ml) and a receiving compartment containing 0.9% w/w NaCl saline
266 solution. To simulate the *stratum corneum* properties a membrane pig ear skin was used. Skin slices
267 were isolated with a dermatome from the outer side of pig ears, obtained from a local slaughterhouse,
268 and then were frozen at $-18\text{ }^{\circ}\text{C}$. Before starting the experiments, the skin was equilibrated in NaCl 0.9
269 % w/w saline solution, in the presence of 0.01% sodium azide to preserve the skin, at $25\text{ }^{\circ}\text{C}$ for 30 min.
270 Then, after washing with saline solution, the skin layer was inserted between the two compartments of
271 the Franz cell, with the stratum corneum side facing towards the donor chamber. The study was carried
272 out for 24 hours and the receiving phase was withdrawn at regular times and replaced with the same
273 amount of fresh receiving medium. The collected samples were then analyzed by HPLC to determine
274 the amount of Vm permeated over time. US abilities to promote Vm permeation were also investigated.
275 For this purpose, a high frequency US transducer ($f = 2.5\text{ MHz}$; $P = 5\text{ W}$; $t = 10\text{ min}$) was combined to
276 a purposely modified vertical diffusion cell. Drug permeation through pig skin after US application was
277 monitored by HPLC analysis of the cumulative amount of antibiotic reaching the receiving phase over
278 time.

279

280 **2.6. Human biocompatibility studies**

281

282 **2.6.1. *Human keratinocyte cell cultures***

283 HaCaT, a long-term cell line of human keratinocytes immortalized from a 62-year old Caucasian male
284 donor (Boukamp et al., 1988), was used for the assessment of Vm and VmLNB biocompatibility. Cells
285 were grown as adherent monolayers in DMEM medium supplemented with 10% fetal bovine serum,
286 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine in a humidified CO₂/air-
287 incubator at 37°C. Before starting the experiments, cells were washed with PBS, detached with
288 trypsin/ethylenediaminetetraacetic acid (0.05/0.02 % v/v), washed with fresh medium and plated at a
289 standard density (10⁶ cells/well in 6-well plates) in 2 mL of fresh medium.

290

291 **2.6.2. *Vm and VmLNB cytotoxicity***

292 The potential cytotoxic effects of VmLNBs were measured as the release of lactate dehydrogenase
293 (LDH) from HaCaT cells into the extracellular medium. Briefly, cells were incubated in DMEM
294 medium for 24 h with/without 1 mg/mL Vm, either free or carried by VmLNBs, in a humidified
295 CO₂/air-incubator at 37°C. Then, 1 mL of cell supernatants was collected and centrifuged at 12000 rpm
296 for 2 min. Cells were washed with fresh medium, detached with trypsin/ethylenediaminetetraacetic acid
297 (0.05/0.02 % v/v), washed with PBS, resuspended in 1 mL of TRAP (82.3 mM triethanolamine, pH
298 7.6), and sonicated on ice with a 10 s burst. 5 µL of cell lysates and 50 µL of cell supernatants were
299 diluted with TRAP and supplemented with 0.5 mM sodium pyruvate and 0.25 mM NADH (300 µL as a
300 final volume) to start the reaction. The reaction was followed measuring the absorbance at 340 nm (37
301 °C) with Synergy HT microplate reader. Both intracellular and extracellular enzyme activities were

expressed as μmol of oxidized NADH/min/well. Finally, cytotoxicity was calculated as the net ratio between extracellular and total (intracellular + extracellular) LDH activities.

2.6.3. Human keratinocyte cell viability

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HaCaT cells were incubated for 24 h with/without 1 mg/mL Vm, either free or carried by VmLNBS, in a humidified CO₂/air-incubator at 37°C. Thereafter, 20 μL of 5 mg/mL MTT in PBS were added to cells for 3 additional hours at 37 °C. The plates were then centrifuged, the supernatants discarded and the dark blue formazan crystals dissolved using 100 μL of lysis buffer containing 20 % (w/v) sodium dodecyl sulfate, 40 % N,N-dimethylformamide (pH 4.7 in 80 % acetic acid). The plates were then read on Synergy HT microplate reader at a test wavelength of 550 nm and at a reference wavelength of 650 nm.

2.7. Microbiological assays

2.7.1. Determination of vancomycin antimicrobial activity against MRSA

Vm solutions were freshly prepared for each experiment. Determination of the minimum inhibitory concentration (MIC) of vancomycin was carried by the microdilution broth method according to the latest Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). Interpretation of the results was performed as outlined in the above mentioned CLSI guidelines (CLSI 2012).

2.7.2. In vitro antibacterial efficiency of VmLNBS against MRSA.

MRSA, isolated from human ulcerated wounds (Infermi Hospital, Biella, Italy), was cultured overnight at 37°C in TSB. After incubation, bacteria were re-suspended in 100 mL of TSB, harvested by 10

326 min centrifugation at 4,000 rpm, diluted in TSB to 10^4 colony-forming-unit (CFU)/mL, as confirmed
327 by colony counts on TSA, and then incubated in TSB with VmLNBs, loaded with Vm at different
328 concentrations (1, 0.1, 0.01, and 0.004 mg/mL), in sterile sampling tubes for 2, 3, 4, 6, and 24 hours at
329 37°C. Controls represented by either bacteria incubated in TSB, bacteria incubated with blank NBs or
330 bacteria incubated in the presence of free Vm at different concentrations (1, 0.1, 0.01 and 0.004
331 mg/mL), were also performed. At each incubation time, serial 10-fold dilutions in saline solution (0.9%
332 NaCl) were prepared from each sample, and 100 μ L of each dilution were spread on TSA, so that the
333 number of CFU/mL could be determined.

334

335 ***2.7.3. Imaging with confocal laser scanning microscopy***

336 MRSA bacteria were grown in TSB at 37°C in agitation until reaching the concentration of 1×10^9
337 CFU/mL. Then, 1 mL aliquot of bacteria was pelleted (3000g x 10 min at 4°C), resuspended in PBS 1x
338 and incubated with 6-coumarin-labeled VmLNBs, 6-coumarin-labeled NBs, or FITC-labeled Vm at a
339 dilution of 1:11, as for previous experiments performed on eukaryotic cells. Each sample was placed on
340 orbital shaker (160 rpm) in the dark at 37°C for 2h and 4h. After incubation, one drop from each
341 suspension was streaked on poly-L-lysine-coated microscope slides and allowed to dry. Then, bacteria
342 were stained with iodide propidium (PI) in PBS 1X and again allowed to dry. Fluorescence images
343 were taken with an Olympus IX70 inverted laser scanning confocal microscope, and captured using
344 FluoView 200 software.

345

346 ***2.8. Statistical analysis***

347 At least three independent experiments, each one in duplicate or triplicate, were performed for every
348 investigational study. Numerical data are shown as means \pm SEM for inferential results or as means \pm
349 SD for descriptive results (see Cumming et al., 2007 for an exhaustive review). Imaging data are shown

350 as representative pictures. All data were analyzed by a one-way Analysis of Variance (ANOVA)
351 followed by Tukey's post-hoc test (software: SPSS 16.0 for Windows, SPSS Inc., Chicago, IL). $P < 0.05$
352 were considered significant.

353

3. Results

3.1. Characterization of VmLNB and control (blank NB and Vm) formulations

Before NB production, the interaction between dextran sulfate and Vm was firstly investigated to optimize Vm/dextran sulfate ratio. Results indicated that Vm was complexed at 99% by dextran sulfate solution until the concentration of 0.5 mg/mL (data not shown). The Vm/dextran sulfate ratio was calculated corresponding to 2:1 (w/w). Based on this preliminary information, NBs were prepared according to the protocol described in the Materials and Methods section. After manufacturing, VmLNB and blank NB formulations (with or without 6-coumarin in the inner core) were characterized physico-chemically. Results are shown in **Figure 2** and **Table 1**. Both VmLNBs and NBs displayed spherical shapes with a core-shell structure by TEM analyses. All sizes were in the nanometer range, with all formulations displaying around 300 nm as a value for average diameters. All polydispersity indexes were included between 0.22 and 0.25. Zeta potentials ranged from -34 mV (NBs) to -29 mV (VmLNBs). The loading of Vm in the NB structure did not significantly affect the viscosity of the formulations. NBs were able to load Vm with an encapsulation efficiency of 86% and loading capacity of 29%.

3.2. Stability of VmLNB and control (blank NB and Vm) formulations

NB and VmLNB formulations proved to be physically stable over time, as confirmed by long-term checking of the parameters assessed in the previous paragraph. Indeed, the obtained values did not remarkably change up to six months after the manufacturing of the formulations stored at 4 °C (data not shown). Furthermore, the chemical stability of the drug was comparatively checked between free Vm solution and VmLNB aqueous suspension either over time (up to 14 days) or at different temperatures (25°C and 37°C). As shown in **Figure 3**, the drug resulted much more stable from a

378 chemical point of view when properly incorporated in the nanocarrier (VmLNBs) than as such in
379 solution.

380

381 **3.3. Human biocompatibility**

382

383 The potential toxicity of Vm solution and VmLNB suspension on human skin cells was assessed by
384 testing *in vitro* cultured HaCaT keratinocytes. Cells were incubated for 24 h alone, with 10% v/v Vm
385 solution, or with VmLNB nanosuspensions in normoxic conditions (20% O₂). Thereafter, cytotoxicity
386 was analyzed by LDH assay, and cell viability by MTT assay. As shown in **Figure 4**, neither Vm nor
387 VmLNBs did show significant toxic effects and HaCaT cell viability was not significantly affected by
388 either formulation.

389

390 **3.4. *In vitro* drug release from VmLNBs**

391 *In vitro* drug release from VmLNB nanosuspension and free Vm solution were comparatively
392 evaluated over time. As shown in **Figure 5** (time course studies up to 6 h) and **Table 2** (end-point data
393 up to 24 h), 1 mg/mL Vm release from VmLNBs was slow and prolonged over time, compared to free
394 drug solution diffusion. No initial burst effect was observed indicating Vm incorporation in NB shell.
395 Further information on additional incubation times and drug concentrations for VmLNBs is available in
396 Supplementary Materials (**Table S3**). Vm/VmLNB drug release ratios at different times (2, 3, 4, 6, and
397 24 h) were also calculated (see **Table 2**), in order to allow normalization of the results from treatment
398 with VmLNBs in the microbiological experiments described in the following paragraph.

399

400

401

3.5. *In vitro* antimicrobial activity of VmLNBs

According to preliminary microbiological analyses performed on the MRSA strain employed in the present experiments, 0.004 mg/mL resulted as the MIC value for Vm. Therefore, decreasing Vm concentrations from 1 mg/mL (used for the studies described in the previous paragraphs) to 0.004 mg/mL (MIC value) were employed in a series of experiments aimed at comparatively evaluating Vm (either free or carried by VmLNBs) antibacterial effectiveness against MRSA. Bacteria were incubated at different times (2, 3, 4, 6, and 24 h) either alone (ctr) or with free Vm, VmLNBs, or blank NBs. The initial drug concentrations (1; 0.1; 0.01; and 0.004 mg/mL) loaded on VmLNBs were the same as those solved in free Vm solution. However, as emerged in the previous paragraph, drug release from VmLNBs is significantly slower than free Vm solution diffusion. For this reason, before proceeding with the analysis of the results, all values on bacterial growth referring to Vm- and VmLNB-treated samples were normalized upon time-dependent Vm/VmLNB drug release ratios shown in **Table 2**. Normalized results are shown in **Figure 6**, whereas raw data are available in Supplementary Materials (**Figure S2**). 1 mg/mL Vm effectively inhibited bacterial growth at all times, independently from being free or carried by the nanocarrier. Lower drug concentrations of free Vm solution were effective against MRSA only after longer times of incubation (at least 3 h for 0.1 mg/mL and 0.01 mg/mL Vm; and at least 4 h for 0.004 mg/mL Vm). Interestingly, Vm antibacterial efficacy was significantly improved when the drug was carried by VmLNBs. Indeed, VmLNB-dependent inhibition of bacterial growth was significantly enhanced compared to free Vm solution, at all drug concentrations. Additionally, compared to free Vm solution, VmLNB antibacterial effects appeared earlier, as they were already evident after 2 h of incubation (the first time-point of the observational period) at all Vm concentrations. Blank NBs did not show any antibacterial activity.

424 Further analysis by confocal microscopy (**Figure 7**) displayed that MRSA avidly internalized free
425 fluorescent Vm already after 2 h of incubation, but not fluorescent VmLNBs. Fluorescent Vm-free NBs
426 did adhere to the bacterial cell wall without being internalized.

427 **3.6. US-triggered drug permeation**

428 The ability of US to promote Vm permeation through the skin was assayed by employing a purposely
429 modified Franz cell constituted by a donor and a recipient chamber separated by a pig skin layer (see
430 **Figure 8A** for a schematic representation of the apparatus). As shown in **Figure 8B**, the administration
431 of US ($t = 10$ min; $f = 2.5$ MHz; $P = 5$ W) strongly induced VmLNBs to deliver the antibiotic drug
432 from the donor chamber throughout the pig skin membrane into the recipient chamber up to 6 h.
433 Furthermore, drug accumulated in the skin after US treatment reached $158 \mu\text{g}/\text{cm}^2$ after 6 hours.

434

435

436

437

4. Discussion

Vm currently represents the main stay against MRSA infections (Koyama et al., 2013; Kullar et al., 2016). However, Vm administration raises several issues that urgently need to be faced, including its marked instability, low oral bioavailability, complex concentration-time profile, low tissue penetration (ranging from 10% in diabetic to 30% in normal skin and soft tissues), and several adverse effects (Mawhinney et al., 1992; Raverdy et al., 2013; Vandecasteele et al., 2012; Vidal et al., 1992; Giandalia et al., 2001). In the attempt to counteract these drawbacks, thus improving the effectiveness of Vm delivery, some novel nanocarriers have been developed: i) Vm coupling to chitosan as an ocular drug delivery vehicle for topical use in rabbit eyes has appeared more effective than carrier-free Vm (Khangtragool et al., 2011); ii) PEGylated liposomal Vm enhanced the effective treatment of MRSA pneumonia and simultaneously reduced the nephrotoxicity risk compared with conventional and non-PEGylated Vm formulations (Muppidi et al., 2011); iii) Vm-loaded liposomes, stabilized with chitosan modified gold nanoparticles bounded to their surface, have proven effective in inhibiting the bacterial growth (Pornpattananangkul et al., 2011); and iv) Vm-containing trehalose and hydroxyethylcellulose spherical matrices have been developed as new delivery systems suitable for topical applications on extensive and purulent wounds (Giandalia et al., 2001). Recently, Vm-loaded polymersomes were developed from a novel pegylated oleic acid polymer for sustained antibiotic delivery (Omolo et al., 2017). Overall, these works represent the proof-of-principle for the feasibility of choice of nanocarriers, as alternative drug delivery systems to obtain the desired drug release rates and bioavailability (Kalhapure et al., 2015). However, the effectiveness of those nanocarriers was seriously undermined by their poor ability to cross the *stratum corneum*, a skin barrier displaying low permeability unless proper exogenous physical stimuli are provided (Azagury et al., 2014; Park et al., 2012).

461 For these reasons, the present study aimed at developing Vm nanocarriers as a new platform to be
462 effectively and safely employed for Vm topical administration to treat wound infections. To this
463 purpose, NBs with core-shell nanostructures were identified as first choice carriers due to their known
464 benefits in association with drug delivery, including small size, stability, suitability for drug loading,
465 responsiveness to external stimuli such as US, and controlled drug release abilities (Marano et al.,
466 2016; Cavalli et al., 2009a; Cavalli et al., 2009b; Cavalli et al., 2016). In this study, dextran sulfate was
467 chosen as main constituent of the polysaccharidic shell as a consequence of the large amount of data
468 from the literature supporting dextran biocompatibility (Bos et al., 2005; De Groot et al., 2001).
469 Encouragingly, dextran-based hydrogels have already been employed as matrices in tissue engineering,
470 without showing signs of inflammation *in vivo* (Möller et al., 2007), and recent toxicological studies
471 have shown that dextran, as well as the products from its mechano-chemical processing, can be
472 classified as class 4 (low-toxicity) substances (Dushkin et al., 2013). Moreover, dextran sulfate presents
473 a negative charge that can electrostatically interact with the positive charged Vm. On the other hand,
474 PFP was employed as principal constituent of the inner core, since it is the most widely used
475 fluorocarbon in oxygenating emulsions and NB formulations (Cabralles and Intaglietta, 2013, Castro
476 and Briceno, 2010). In order to load Vm, dextran sulfate-shelled/PFP-cored NBs were then
477 functionalized by exploiting the electrostatic interactions occurring between the negatively charged
478 sulfate groups of the shell and the protonated amino groups of the drug. The obtained VmLNBS
479 displayed a spherical shape and a well-defined core-shell structure with a polymeric shell thickness of
480 about 40 nm, average diameters of 300 nm, viscosity of 1.25 cP, and negatively charged surfaces. Of
481 note, the observed decrease of zeta potential values of ~ 15 % for VmLNBS (around -29 mV) with
482 respect to blank NBs (around -34 mV) confirmed the occurrence of electrostatic interactions between
483 positive amino groups of the drug and negative sulfate groups of the polymer, leading to a partial

charge neutralization of the bubble surface and allowing a good Vm encapsulation efficiency (86%) and loading capacity (29%) in the NB systems.

In addition, it should be noticed that since the zeta potential measures charge repulsion or attraction between particles, it represents a fundamental parameter to avoid nanoparticle aggregation, with zeta potentials lower than -25 mV or larger than +25 mV being generally required for physical stability of colloid systems (Shah and Eldridge, 2014). The stability of VmLNB formulations was further confirmed by long-term checking of their size, surface charge, and viscosity values, which did not show any significant changes up to six months after manufacturing, stored at 4 °C. On the other hand, drug stability was comparatively checked between free Vm solution and VmLNB suspension either over time (up to 14 days) or at different temperatures (25°C and 37°C), revealing an increased stability for Vm when properly encapsulated in the nanocarriers. This appears as an undoubtedly advantageous feature for VmLNB formulations, since they might prove useful to overcome the reported instability of Vm in aqueous solutions at body temperature (Mawhinney et al., 1992, Raverdy et al., 2013). Interestingly, VmLNBs displayed a slow and prolonged drug release kinetics compared to Vm aqueous solution, with only 16% of the drug being released from VmLNBs after 6 h. These data support the hypothesis that VmLNBs may be employed as an effective drug reservoir until reaching the target site, where the antibiotic would be released upon sonication at an appropriate moment only. The features of VmLNBs might be exploited for the design of innovative wound dressing following their inclusion in polymeric base. Indeed, NBs can be dispersed in polymer gel without changing physico-chemical characteristics, as previously showed (Prato et al., 2015). Another intriguing feature of VmLNBs relies on the reported evidence that surface charges play a pivotal role in making a nanoparticle suitable for topical treatment, since they enhance its interaction with the skin and improve its therapeutic effect on inflamed cutaneous tissues, either without (Abdel-Mottaleb et al., 2012) or with concomitant US treatment (Lopez et al., 2011). Although cationic nanoparticles are generally preferred for topical

508 treatment due to the anionic nature of the skin (Wu et al., 2010), some authors have shown that anionic
509 nanoparticles can be more effective (Lee et al., 2013) and less toxic (Ryman-Rasmussen et al., 2007)
510 than the cationic ones. These latter data appear consistent with our results through investigation by
511 biochemical assays to assess VmLNB biocompatibility with human skin tissues. Indeed, VmLNBs did
512 not induce any *in vitro* cytotoxic effects on HaCaT keratinocytes, a skin cell line that was originally
513 immortalized from a 62-year old donor (Boukamp et al., 1988). This peculiar information strengthens
514 remarkably the evidence on VmLNB safety for future topical applications.

515 VmLNB and carrier-free Vm antimicrobial activity against MRSA were comparatively investigated,
516 also analyzing Vm and NB physical interaction with the bacterial cell wall by confocal microscopy.
517 Interestingly, VmLNBs were more effective in MRSA bacterial growth inhibition than free Vm,
518 promoting enhanced and earlier antibacterial effects, although they were not internalized by bacteria,
519 opposite to free Vm. This behavior appears to be a likely consequence of time-sustained release of Vm
520 from VmLNBs.

521 Notably, an important issue that requires caution while evaluating the feasibility for any topical drug
522 treatment is represented by the considerably low degree of permeability of the skin, the primary
523 defense system for the body. This organ consists of several layers, including the *stratum corneum*, the
524 epidermis, and the dermis. In particular the *stratum corneum* - composed of corneocytes interspersed in
525 a laminate of compressed keratin and intercorneocyte lipid lamellae - is very poorly permeable to
526 foreign molecules and represents the main obstacle to transdermal drug delivery (Naik et al., 2000).
527 However, an ideal antibiotic drug formulation should be efficiently localized in the epidermis/dermis
528 and provide a sustained drug release over time (Prabhu et al., 2012). To allow a drug to penetrate the
529 skin, several approaches have been proposed, including skin patches, ionophoresis, chemical
530 enhancers, and US-triggered sonophoresis (Park et al., 2014).

531 Interestingly, antimicrobial properties have been reported for US, although its effectiveness strongly
532 varies depending on the targeted type of pathogen (fungi vs bacteria; cocci vs bacilli; Gram-positive vs
533 Gram-negative) (Sango et al., 2014). Furthermore, synergistic effects between US and antibiotics have
534 been reported in a series of studies: i) antibiotic treatment coupled with US irradiation resulted in
535 enhanced bactericidal activity against both Gram-positive and Gram-negative bacteria, especially for
536 aminoglycosides (Yu et al., 2012); ii) the combination of Vm and US decreased *S. aureus* viable counts
537 by two orders of magnitude compared to Vm alone (Ayan et al., 2008); and iii) the addition of NB-
538 enhanced US to doxycycline treatment improved the drug effectiveness in eradicating intracellular
539 *Chlamydia trachomatis* (Ikeka-Dantsuji et al., 2011). US-dependent enhancement of antibiotic action
540 on biofilms was named as a ‘bioacoustic effect’. Interestingly, Vm transfer through *S. epidermidis*
541 biofilms was shown to be significantly enhanced by US, with bubbles being able to increase the biofilm
542 permeability to Vm (Dong et al., 2013).

543 As discussed previously, VmLNBs can be effectively employed as an important reservoir to store the
544 drug until trespassing the *stratum corneum* of the skin and reaching the target site. In order to achieve
545 the latter goal, US was assayed for its ability to induce VmLNBs to trespass an *in vitro* cutaneous layer,
546 thereby releasing Vm throughout the skin. Notably, the skin from the pig ear is widely recognized as a
547 good model for human skin permeability, since it displays human-like histological and physiological
548 properties, including epidermal thickness and composition, dermal structure, lipid content and general
549 morphology (Dick and Scott, 1992). The validity of the porcine model has been established by
550 comparing the permeability of simple marker molecules with the corresponding values across human
551 skin (Herkenne et al., 2006, Sekkat et al., 2002). Therefore, the porcine ear skin represents so far the
552 most accountable *in vitro* model to mimic the human skin in studies on percutaneous penetration
553 (Jacobi et al., 2007). In our experiments, US appeared essential to promote Vm release from VmLNBs
554 throughout the pig skin layers, in line with previous reports on NBs and sonophoresis. On the contrary,

555 the passive transport of free vancomycin hydrochloride was negligible, being a charged and hydrophilic
556 molecule. The amount of Vm accumulated in the skin after US application combined with NBs was
557 greater than MIC value.

558

559 **5. Conclusions**

560 In the present work, dextran sulfate-shelled and PFP-filled NBs were developed for Vm delivery.
561 VLNBs proved to be effective in MRSA bacterial killing without showing toxic effects on human
562 keratinocytes. The combination of NBs and US enhanced Vm permeation through pig skin and
563 promoted drug skin accumulation. Based on these results, Vm topical administration through proper
564 NB formulations might be a promising strategy for the local treatment of MRSA skin infections. The
565 study represents the proof of concept for the future development of advanced multifunctional
566 therapeutic systems to treat infected wounds.

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830 **Figure legends**

831

832 **Figure 1. Schematic structure of VmLNB formulations.** Vm nanocarriers described in the present
833 work display a core-shell structure. PFP was employed as core fluorocarbon, whereas dextran sulfate
834 was chosen as polysaccharidic shell molecule. Vm was inserted into the outer shell throughout dextran
835 sulfate chains. In selected experiments, VmLNBs were further functionalized by including fluorescent
836 6-coumarin in the inner core.

837

838 **Figure 2. NB and VmLNB morphology.** NBs and VmLNBs were checked for morphology by TEM.
839 Results are shown as representative images from three different preparations. Panel A. NB image by
840 TEM. Panel B. VmLNB image by TEM. (see also Figure S1 in Supplementary Materials for additional
841 images of multiple nanobubbles within the same field).

842

843 **Figure 3. Stability of Vm and VmLNB formulations.** The stability of Vm solution and VmLNB
844 suspension was monitored up to 14 days either at room temperature (Panel A) or at 37°C (Panel B)
845 through analysis by HPLC. Results are shown as means \pm SD from three different preparations for each
846 formulation. Data were also analyzed for significance by ANOVA. Versus Vm solution: * $p < 0.001$.

847

848 **Figure 4. Biocompatibility of Vm and VmLNBs with human keratinocytes *in vitro*.** HaCaT cells
849 (10^6 cells/2 mL DMEM medium implemented with 10% FCS) were left untreated (ctr) or treated with
850 200 μ L of Vm solution or VmLNB suspension for 24 h in normoxia (20% O₂). Thereafter, Vm and
851 VmLNB cytotoxicity were measured through LDH assay (Panel A), whereas cell viability was
852 measured through MTT assay (Panel B). Results are shown as means \pm SEM from three independent

853 experiments. Data were also evaluated for significance by ANOVA. No significant differences were
854 found among all conditions.

855 **Figure 5. *In vitro* Vm release from Vm and VmLNB formulations.** Vm release from Vm solution
856 and VmLNB suspension was monitored up to 6 h. Results are shown as means \pm SD from three
857 different preparations for each formulation. Data were also analyzed for significance by ANOVA.
858 Versus Vm solution: * $p < 0.001$.

859

860 **Figure 6. Antibacterial activity of Vm and VmLNBS against MRSA.** MRSA were left for 2, 3, 4, 6
861 and 24 hours at 37°C alone (ctr) or incubated with 10% v/v NBs or different concentrations of Vm,
862 either free or loaded on VmLNBS (Panel A: 1 mg/mL; Panel B: 0.1 mg/mL; Panel C: 0.01 mg/mL;
863 Panel D: 0.004 mg/mL). Results are shown as means \pm SEM from three independent experiments. Data
864 on Vm- and VmLNB-treated samples were normalized upon Vm/VmLNB release ratios reported in
865 Table 2 (see also in Supplementary Materials: Table S3 for further information on percentages of drug
866 release from VmLNBS at different times/concentrations; and Figure S2 for raw data on VmLNB
867 antibacterial effects). All data were also evaluated for significance by ANOVA. Versus ctr: * $p < 0.02$;
868 versus Vm: ° $p < 0.05$.

869

870 **Figure 7. Drug loading on dextran sulfate-shelled NBs prevents Vm internalization by MRSA.**
871 MRSA were left alone or incubated with 10% v/v 6-coumarin-labeled VLNBS, 6-coumarin-labeled
872 NBs, and FITC-labeled Vm for 2h at 37°C. After staining bacteria with PI, confocal fluorescent images
873 were taken using FITC and TRITC filters. Data are shown as representative images from three
874 independent experiments. Magnification: 100X. Red: PI. Green: 6-coumarin or FITC.

875

876 **Figure 8. US-triggered sonophoresis of VmLNBs through skin membranes.** US ($t = 10$ min; $f = 2.5$
877 MHz; $P = 5$ W) abilities to induce sonophoresis and Vm permeation from VmLNBs were evaluated up
878 to 6 h by using a vertical diffusion Franz cell consisting in two chambers (donor and recipient,
879 respectively) separated by a pig skin layer (see scheme in Panel A). Results are shown in Panel B as
880 means \pm SD from three independent experiments. Data were also evaluated for significance by
881 ANOVA. Versus without US: $p < 0.001$.

882 **Tables and legends**

883

Formulation	Average diameter \pm SD (nm)	Polydispersity index	Zeta Potential \pm SD (mV)	Viscosity (cP)
NBs	313.4 \pm 26.4	0.24 \pm 0.02	- 34.5 \pm 0.38	1.22
VmLNBs	304.6 \pm 14.6	0.22 \pm 0.03	- 28.6 \pm 1.34	1.25
Fluorescent NBs	312.8 \pm 22.7	0.25 \pm 0.02	- 34.1 \pm 1.22	1.24
Fluorescent VmLNBs	308.9 \pm 22.4	0.23 \pm 0.01	- 29.5 \pm 1.88	1.23

884

885 **Table 1. Physical-chemical characterization of NBs and VmLNBs.** Liquid formulations were
886 characterized for average diameters, polydispersity index, and zeta potential by light scattering. The
887 viscosity (cP) of NB and VmLNB suspensions was determined at 25 °C by using a Ubbelohde capillary
888 viscosimeter. Results are shown as means \pm SD from three preparations. See also Figures 1-2 for
889 further detail on NB and VmLNB structure and morphology.

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time (hours)	% drug release from Vm solution	% drug release from VmLNBs	Vm/VmLNB drug release ratio
2	36.57	5.99	6.11
3	45.97	7.97	5.78
4	57.16	10.27	5.57
6	73.44	14.59	5.03
24	92.34	35.84	2.58

898

899 **Table 2. *In vitro* drug release from Vm solution and VmLNB suspension.** After incubation for
900 increasing times (first column), the percentages of *in vitro* drug release from Vm solution (second
901 column) and VmLNB suspension (third column) were measured. Then, Vm/VmLNB drug release
902 ratios (fourth column) were calculated for each time considered. All incubation times (2, 3, 4, 6, and
903 24 h) were purposely chosen to further normalize the results from the experiments with MRSA (see
904 Figure 6). Results are shown as mean values from three different preparations for each formulation.

1 **Vancomycin-loaded nanobubbles: a new platform for controlled antibiotic delivery against**
2 **methicillin-resistant *Staphylococcus aureus* infections.**

3
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23 **Abstract**

24

25 Vancomycin (Vm) currently represents the gold standard against methicillin-resistant *Staphylococcus*
26 *aureus* (MRSA) infections. However, it is associated with low oral bioavailability, formulation stability
27 issues, and severe side effects upon systemic administration. These drawbacks could be overcome by
28 Vm topical administration if properly encapsulated in a nanocarrier. Intriguingly, nanobubbles (NBs)
29 are responsive to physical external stimuli such as ultrasound (US), promoting drug delivery. In this
30 work, perfluoropentane (PFP)-cored NBs were loaded with Vm by coupling to the outer dextran sulfate
31 shell. Vm-loaded NBs (VmLNBs) displayed ~300 nm sizes, anionic surfaces and good drug
32 encapsulation efficiency. *In vitro*, VmLNBs showed prolonged drug release kinetics, not accompanied
33 by cytotoxicity on human keratinocytes. Interestingly, VmLNBs were generally more effective than
34 Vm alone in MRSA killing, with VmLNB antibacterial activity being more sustained over time as a
35 result of prolonged drug release profile. Besides, VmLNBs were not internalized by *staphylococci*,
36 opposite to Vm solution. Further US association promoted drug delivery from VmLNBs through an *in*
37 *vitro* model of porcine skin. Taken together, these results support the hypothesis that proper Vm
38 encapsulation in US-responsive NBs might be a promising strategy for the topical treatment of MRSA
39 wound infections.

40

41 **Key words**

42 Nanobubbles; vancomycin; methicillin-resistant *Staphylococcus aureus*; ultrasound; prolonged release.

43

44 **1. Introduction**

45

46 Chronic wounds fail to proceed through timely regulated and interrelated processes to restore
47 anatomical and functional integrity of the injured tissues (Lazarus et al., 1994) such as diabetic feet,
48 bedsores, and venous ulcers (Markova et al., 2012). To date, these types of wounds are considered like
49 a silent epidemic, affecting a large fraction of the world population and posing a major gathering threat
50 to the public health and economy of all developed countries (Daeschlein, 2013). Hospitalized patients
51 are at particular risk, especially those suffering from diabetes, human immunodeficiency virus or other
52 immune disorders, as well as those undergoing chemotherapy (Payne et al., 2008).

53 Beyond delayed healing processes due to different factors (hypoxia, persistent inflammation, and
54 altered balances between tissue remodelling proteinases and their inhibitors), chronic wounds are often
55 worsened by microbial infections (Gurusamy et al., 2013). Among the bacteria responsible for skin
56 infection, *Staphylococcus aureus* represents the most common pathogen to be identified in chronic
57 wounds, with methicillin-resistant *S. aureus* (MRSA) accounting for upward of 20% to 50% of cases
58 (Price, 2010). MRSA colonies often develop at the interface between synthetic prostheses and
59 biological tissues, particularly during surgery and post-surgery course. In addition, MRSA colonization
60 or infection of wounds can result in MRSA bacteremia, which is associated with a 30-day mortality of
61 about 28% to 38% patients (Gurusamy et al., 2013).

62 The main goal of chronic wound treatment is to decrease the injuring-associated microbial load, thus
63 allowing wound healing processes to take place. However, conventional systemic delivery of
64 antibiotics not only entails poor penetration into ischemic and necrotic tissues, but can also cause
65 systemic toxicity with associated renal and liver complications, resulting in forced hospitalization for
66 further monitoring and advanced treatment. On the contrary, topically applied antimicrobials have

67 | proven effective in decreasing bacterial levels in granulating wounds (Diehr et al., 2008~~7~~). Therefore,
68 | alternative local delivery of antimicrobials - either by topical administration or through novel delivery
69 | devices - may enable to keep high local antibiotic concentrations for prolonged release times without
70 | reaching systemic toxicity (Zilberman et al., 2008).

71 | A promising approach to develop a topical therapy for microbial infection in skin and soft tissues
72 | would employ biocompatible nanomaterials and drug nanocarriers. Indeed, nanotechnology represents
73 | an emerging field to be exploited for antibiotic drug delivery. Thanks to their physical and chemical
74 | properties (small size, high surface-to-volume ratio and suitable surface modification) nano-sized
75 | materials may be used as drug carriers to trespass several physiological barriers and to reach biological
76 | targets. The coupling of nanocarriers with anti-infectious agents makes it likely to increase drug
77 | concentrations and drug penetration at the site of infection. As a result, it might not only improve the
78 | therapeutic index but also reduce some issues associated with nonspecific cytotoxicity and antibiotic
79 | resistance (Sharma et al., 2012).

80 | Vancomycin hydrochloride, being effective against many Gram-positive bacteria that are unresponsive
81 | to common antibiotics, represents the gold standard against MRSA infections (Kullar~~ant~~ et al., 2016).
82 | However, Vm is poorly absorbed from the gastrointestinal tract with a low oral bioavailabiliy. Low
83 | intravenous infusion is often suggested as a feasible alternative for drug administration, but Vm
84 | instability in aqueous solutions at 37°C could imply a tremendous reduction of drug effectiveness
85 | (Mawhinney et al., 1992; Raverdy et al., 2013). Following parenteral administration, Vm displays a
86 | slow mode of action, a complex concentration-time profile, and a disappointingly low penetration in
87 | tissues (Vandecasteele et al., 2012). Furthermore, systemic Vm administration can be associated with
88 | several adverse effects (Vidal et al., 1992). On the other hand, Vm topical application – that would be
89 | much safer than systemic administration - is currently limited by several factors such as skin barrier
90 | properties and poor drug permeability (Giandalia et al., 2001). Being the main goal of chronic wound

91 treatment to decrease the microbial load, allowing the healing processes to take place, new delivery
92 protocol should be devised, since conventional systemic delivery of antibiotics requires a drug
93 concentration which is locally ineffective because of the poor penetration into ischemic and necrotic
94 tissues, but can cause systemic toxicity and topically applied antimicrobials have proven effective in
95 decreasing bacterial levels in granulating wounds (Diehr et al., 2007), without inducing systemic
96 toxicity (Zilberman et al., 2008) but suffer from poor diffusion across membranes.

97 Intriguingly, the use of a nanocarrier may help to avoid the abovementioned drawbacks. Notably,
98 nanocarriers such as liposomes, microemulsions, and lipid nanoparticles have the potential to deliver
99 drugs to the skin more efficiently than conventional topical carriers such as creams and ointments, that
100 are not usually recommended for applications on injured skin (Giandalia et al., 2001; Prabhu et al.,
101 2012). However, the response to drug topical applications has been too weak so far, mainly due to the
102 inability to cross the external skin barrier (*stratum corneum*) and reach the dermal regions where the
103 bacteria are nested. Interestingly, physical media such as ultrasound (US) are reportedly able to trigger
104 drug release at the site of infection by temporarily increasing skin permeability through sonophoresis.
105 As such, US is useful to promote drug targeting and transdermal delivery in a non-invasive manner
106 (Azagury et al., 2014; Park et al., 2012).

107 Microbubbles (MBs) (Guiot et al., 2006), nanobubbles (NBs) (Cavalli et al., 2009a; Cavalli et al.,
108 2009b; Cavalli et al., 2016) and nanodroplets (NDs) (Magnetto et al., 2014; Prato et al., 2015) are
109 suitable carriers to be combined with such a physical trigger. They are spherical core-shell structures
110 filled with gases such as perfluorocarbons. Particularly, oxygen-cored nanostructures can be employed
111 both for sonography (as contrast agents) (Fokong et al., 2012; Marxer et al., 2011) and for therapy (as
112 hypoxia- and infection-counteracting devices) (Gulino et al., 2015; Banche et al., 2015; Khadjavi et al.,
113 2015; Basilico et al., 2015; Prato et al., 2016). In particular NBs, consisting in an outer shell of a
114 biocompatible/biodegradable polysaccharide (chitosan, dextran, or dextran sulfate) and an inner core

filled with an oxygen-storing fluorocarbon (perfluoropentane, PFP), have been purposely developed as a new non-invasive, low-cost and multipurpose nanotechnological platform (Cavalli et al., 2009a; Cavalli et al., 2009b; Cavalli et al., 2016). PFP is a perfluorocarbon with a boiling point of 29°C, hence liquid at room temperature. The use of PFP allows liquid droplet generation at room temperature. Then, PFP in nanodroplets can be activated by an external stimulus, like US, by means of a mechanism called acoustic droplet vaporization, causing the droplet to become a bubble. Depending on the properties of the nanostructure, NBs can be subsequently coupled with different molecules, such as drugs or genetic materials, thus acting as nanocarriers (Cavalli et al., 2012; Cavalli et al., 2013; Delalande et al., 2012; Yin et al., 2014). Due to their structure and their gaseous core, NBs are very responsive to US and can take advantage from a number of effects related to microcavitation and microstreaming, occurring at the liquid-membrane interface and responsible for transitory and reversible openings of the pores, thus crossing the membrane itself and delivering their content beyond the tissue (sonophoresis) or the cell (sonoporation) membrane (Karshafian et al., 2009).

Based on these preconditions, the present work aimed at producing dextran sulfate-shelled and PFP-cored NBs for Vm local delivery to potentially treat skin infectious diseases. The formulation is referred to as “nanobubbles” for sake of simplicity but it must be said that, prior to the application of US, it would be more accurate to use the term “nanodroplets” when the core is constituted of PFP.

Therefore, Vm-loaded NBs (VmLNBs) were prepared and characterized for physico-chemical parameters and drug release kinetics; tested for biocompatibility with human skin cells and for their antibacterial properties or interactions with MRSA; and challenged for responsiveness to US, in order to assess their effectiveness as Vm nanocarriers for local delivery.

137 2. Material and methods

138

139 2.1. Materials

140 All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows. Sterile
141 plastics were from Costar, Cambridge, UK; ethanol (96%) was from Carlo Erba (Milan, Italy); soybean
142 lecithin (Epikuron 200®) was from Cargill (Hamburg, Germany); 1-800 Millipore system to obtain
143 ultrapure water and Amicon® Ultra-0.5 centrifugal filter device were from Millipore (Molsheim,
144 France); Ultra-Turrax SG215 homogenizer was from IKA (Staufen, Germany); RPMI 1640 medium
145 was from Invitrogen (Carlsbad, CA); Nanobrook 90Plus Particle Size Analyzer was from Brookhaven
146 (New York City, NY); Philips CM10 electron microscope was from Philips (Eindhoven, the
147 Netherlands); Ubbelohde capillary viscosimeter was from SCHOTT Instruments GmbH (Mainz,
148 Germany); Perkin Elmer PUMP 250B was from Perkin Elmer (Waltham, MA); Flexar UV/Vis LC
149 spectrophotometer detector was from Perkin Elmer (Waltham, MA); Agilent TC C₁₈ columns were
150 from Agilent (Santa Clara, CA); Orion Model 420A pH Meter was from Thermo Scientific (Waltham,
151 MA); Semi-Micro Osmometer K-7400 was from Knauer (Berlin, Germany); Beckman Coulter Allegra
152 64R Centrifuge was from Beckman Coulter (Brea, CA); Spectra/Por cellulose membranes were from
153 Spectrum Laboratories (Rancho Dominguez, CA); HaCaT cells were from Cell Line Service GmbH
154 (Eppelheim, Germany); cell culture RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM)
155 were from Invitrogen (Carlsbad, CA); streptomycin was from Cambrex Bio Science (Vervies,
156 Belgium); humidified CO₂/air-incubator was from Thermo Fisher Scientific Inc. (Waltham, MA);
157 tryptic soy broth (TSB) and tryptic soy agar (TSA) were from Merk KgaA (Darmstadt, Germany);
158 Olympus Fluoview 200 laser scanning confocal system mounted on an inverted IX70 Olympus
159 microscope was from Olympus America Inc. (Melville, NY, USA) ; SPSS 16.0 software was from
160 SPSS Inc. (Chicago, IL).

161

162 **2.2. Development and manufacturing of formulations**

163

164 **2.2.1. Determination of Vm and dextran sulfate interaction ratio**

165 Increasing concentrations (0.25, 0.5, 1.0, 2.0 mg/mL) of dextran sulfate aqueous solutions (1 mL) were
166 added to 1 mL of Vm aqueous solution (1 mg/mL) under magnetic stirring at room temperature
167 overnight. After equilibration, the systems were separated by centrifugation (20000 rpm, 15 minutes)
168 using a centrifugal filter device (Amicon® Ultra), in order to determine the amount of unbound Vm in
169 the filtrate phase. The drug concentration in the filtrate was determined using the HPLC method
170 described below.

171

172 **2.2.2. Preparation of NB, Vm, and VmLNB formulations**

173 NBs were formulated using PFP for the inner core and dextran sulfate for the shell. A purposely tuned
174 multi-step protocol was designed. Briefly, a pre-emulsion was obtained adding 300 μmL of an ethanol
175 solution containing Epikuron® 200 and palmitic acid (1% w/v) to 500 μL of PFP under magnetic
176 stirring. After the addition of 4.8 mL of ultrapure water, the system was homogenized using a Ultra-
177 Turrax SG215 homogenizer. To obtain the polymeric NBs, 350 μL of 1% w/v dextran sulfate
178 (molecular weight = 100 kDa) aqueous solution was added drop-wise under magnetic stirring. Blank
179 NBs obtained according to this procedure were employed as control formulations in the subsequent
180 experiments. On the other hand, to obtain VmLNBs, an extra step based on drop-wise addition of a Vm
181 aqueous solution (pH 3.5) to the so-formed NBs was performed under mild stirring. Different
182 concentrations of Vm solutions were added to prepare a series of VmLNB formulations with increasing
183 drug content (0.004, 0.01, 0.1, and 1 mg/mL). VmLNBs were then purified by dialysis to eliminate

184 unbound molecules. For selected experiments, fluorescent NBs and VmLNBs were obtained by the
185 addition of 6-coumarin (1 mg/mL) to the PFP core. Alternatively, fluorescent Vm was synthesized
186 through reaction between fluorescein isothiocyanate (FITC) and Vm. For this purpose, an amount of
187 FITC solution in methanol (0.2 % w/v) was added to Vm aqueous solution and incubated under stirring
188 overnight in the dark. **Figure 1** shows a representative scheme resuming the general structure of
189 fluorescent VmLNBs. For cell experiments, NBs were prepared in phosphate buffer saline pH 7.4
190 (PBS). For *in vitro* permeation studies, NBs were prepared in saline solution (NaCl 0.9% w/v).

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192 **2.2.3. NB sterilization**

193 Firstly, the glassware and the components were sterilized at 121 °C and 2 bar. Subsequently, all NB
194 formulations were sterilized through UV-C exposure for 20 min. Thereafter, UV-C-treated materials
195 were incubated with cell culture RPMI 1640 medium in a humidified CO₂/air-incubator at 37°C up to
196 72 h, not displaying any signs of microbial contamination when checked by optical microscopy.

198 **2.3. Characterization of formulations**

200 **2.3.1. Characterization of NB and VmLNB formulations**

201 The average diameter, polydispersity index and zeta potential were determined by photocorrelation
202 spectroscopy using a particle size analyzer at a scattering angle of 90° and a temperature of 25 °C. NB
203 suspensions were diluted in deionized filtered water before measurement. For zeta potential
204 determination, samples of diluted NB formulations were placed in the electrophoretic cell, where an
205 electric field of approximately 15 V/cm was applied. The morphology of formulations was evaluated
206 by Transmission Electron Microscopy (TEM), using a Philips CM10 (Eindhoven, NL) instrument. NB
207 and VmLNB aqueous suspensions were sprayed on Formvar-coated copper grid and air-dried before

208 observation. The viscosity of the samples was determined at 25 °C using a Ubbelohde capillary
209 viscosimeter.

210

211 **2.3.2. HPLC quantitative Vm determination**

212 Vm quantitative determination was carried out by using an HPLC system based on a Perkin Elmer
213 pump equipped with a spectrophotometer detector. Analyses were performed using an Agilent TC C₁₈
214 column (250 mm × 4.6 mm, 5 µm). The mobile phase was a mixture of KH₂PO₄ 50 mM (pH 4) and
215 acetonitrile (92:8 v/v), degassed and pumped through the column with a flow rate of 1 mL/min.
216 Ultraviolet detection was set at 286 nm. The external standard method was used to calculate the drug
217 concentration. For this purpose, 1 mg of Vm was weighted, placed in a volumetric flask, and dissolved
218 in water to obtain a stock standard solution. This solution was then diluted using the mobile phase,
219 providing a series of calibration solutions, subsequently injected into the HPLC system. Linear
220 calibration curve was obtained over the concentration range of 0.5–25 µg/mL, with a regression
221 coefficient of 0.999.

222

223 **2.3.3. In vitro evaluation of Vm stability**

224 Vm chemical stability - either solved in aqueous solution or loaded in VmLNBS - was evaluated at
225 room temperature and at 37 °C over time. A quantitative determination of Vm concentration over time
226 was carried out using the HPLC method described above.

227

228 **2.3.4. NB stability over time and after US administration**

229 The physical stability of NBs was evaluated by morphological analysis and by size and zeta potential
230 determination of formulation over time. Their average diameters, zeta potential values and morphology
231 were assessed up to six months. Stability was also investigated following NB exposure to US ($f = 2.5 \pm$

0.1 MHz; t = 10 min; P = 5 W). NB morphology was observed by TEM to confirm the integrity of NB structure.

2.3.5. Encapsulation efficiency and loading capacity of Vm in NBs

The encapsulation efficiency of VmLNBs was determined using a centrifugal filter system. 150 μ mL of VmLNB suspension were put in an Amicon® Ultra-0.5 centrifugal filter device and centrifuged at 15000 rpm for 30 minutes using Beckman Coulter Allegra 64R Centrifuge. The solution filtered in the bottom of the tube was quantified and after suitable dilution was analyzed by HPLC, in order to obtain the concentration of free Vm in VmLNBs suspensions. The encapsulation efficiency was calculated by subtracting the amount of free drug from the initial amount of added Vm, according to the following equation:

$$\text{Encapsulation efficiency} = \frac{(\text{total Vm} - \text{free Vm})}{\text{total Vm}} \times 100$$

The loading capacity was determined on freeze-dried NB samples. Briefly, a weighted amount of freeze-dried VmLNBs was ~~suspended~~ ~~diluted~~ in 105 mL of water. After sonication and centrifugation, the supernatant was ~~diluted with mobile phase and~~ analyzed by HPLC. The loading capacity of Vm in VmLNBs was calculated as follows:

$$\text{Loading capacity} = \frac{(\text{total Vm} - \text{free Vm})}{\text{NB weight}} \times 100$$

2.4. In vitro release studies

In vitro drug release experiments were conducted in a multi-compartment rotating cell, comprising a donor chamber separated by a cellulose membrane (cut-off = 12000 Da) from a receiving compartment. One ml of VmLNB suspension at different concentrations (1, 0.1, 0.01 and 0.004 mg/mL) was placed

254 in the donor chamber. The *in vitro* release kinetics of Vm from VmLNB was compared to a Vm
255 aqueous solution (1 mg/mL) as a control. The receiving phase, containing phosphate buffer 0.05 M (pH
256 7.4) was withdrawn at regular intervals and replaced with the same amount of fresh buffer. Quantitative
257 determination of Vm in the withdrawn samples was carried out by the HPLC method, as described in
258 the previous paragraph. Data were expressed as % of Vm released over time.

259

260 **2.5. *In vitro* permeation study**

261 *In vitro* studies were performed using a vertical diffusion Franz cell to evaluate Vm permeation
262 throughout the skin. The Franz cell consists of a donor compartment, with Vm (1 mg/mL, either free or
263 carried by VmLNBs, 1x10¹² NBs/ml) and a receiving compartment containing 0.9% w/w NaCl saline
264 solution. To simulate the *stratum corneum* properties a membrane pig ear skin was used. Skin slices
265 were isolated with a dermatome from the outer side of pig ears, obtained from a local slaughterhouse,
266 and then were frozen at -18 °C. Before starting the experiments, the skin was equilibrated in NaCl 0.9
267 % w/w saline solution, in the presence of 0.01% sodium azide to preserve the skin, at 25 °C for 30 min.
268 Then, after washing with saline solution, the skin layer was inserted between the two compartments of
269 the Franz cell, with the stratum corneum side facing towards the donor chamber. The study was carried
270 out for 24 hours and the receiving phase was withdrawn at regular times and replaced with the same
271 amount of fresh receiving medium. The collected samples were then analyzed by HPLC to determine
272 the amount of Vm permeated over time. US abilities to promote Vm permeation were also investigated.
273 For this purpose, a high frequency US transducer ($f = 2.5$ MHz; $P = 5$ W; $t = 10$ min) was combined to
274 a purposely modified vertical diffusion cell. Drug permeation through pig skin after US application was
275 monitored by HPLC analysis of the cumulative amount of antibiotic reaching the receiving phase over
276 time.

277

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278 **2.6. Human biocompatibility studies**

279

280 **2.6.1. Human keratinocyte cell cultures**

281 HaCaT, a long-term cell line of human keratinocytes immortalized from a 62-year old Caucasian male
282 donor (Boukamp et al., 1988), was used for the assessment of Vm and VmLNB biocompatibility. Cells
283 were grown as adherent monolayers in DMEM medium supplemented with 10% fetal bovine serum,
284 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine in a humidified CO₂/air-
285 incubator at 37°C. Before starting the experiments, cells were washed with PBS, detached with
286 trypsin/ethylenediaminetetraacetic acid (0.05/0.02 % v/v), washed with fresh medium and plated at a
287 standard density (10⁶ cells/well in 6-well plates) in 2 mL of fresh medium.

288

289 **2.6.2. Vm and VmLNB cytotoxicity**

290 The potential cytotoxic effects of VmLNBs were measured as the release of lactate dehydrogenase
291 (LDH) from HaCaT cells into the extracellular medium. Briefly, cells were incubated in DMEM
292 medium for 24 h with/without 1 mg/mL Vm, either free or carried by VmLNBs, in a humidified
293 CO₂/air-incubator at 37°C. Then, 1 mL of cell supernatants was collected and centrifuged at 12000
294 rpm+3000g for 2 min. Cells were washed with fresh medium, detached with
295 trypsin/ethylenediaminetetraacetic acid (0.05/0.02 % v/v), washed with PBS, resuspended in 1 mL of
296 TRAP (82.3 mM triethanolamine, pH 7.6), and sonicated on ice with a 10 s burst. 5 µmicroL of cell
297 lysates and 50 microµL of cell supernatants were diluted with TRAP and supplemented with 0.5 mM
298 sodium pyruvate and 0.25 mM NADH (300 µmicroL as a final volume) to start the reaction. The
299 reaction was followed measuring the absorbance at 340 nm (37 °C) with Synergy HT microplate
300 reader. Both intracellular and extracellular enzyme activities were expressed as µmol of oxidized

301 NADH/min/well. Finally, cytotoxicity was calculated as the net ratio between extracellular and total
302 (intracellular + extracellular) LDH activities.

303

304 **2.6.3. Human keratinocyte cell viability**

305 Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
306 (MTT) assay. HaCaT cells were incubated for 24 h with/without 1 mg/mL Vm, either free or carried by
307 VmLNBS, in a humidified CO₂/air-incubator at 37°C. Thereafter, 20 ~~micro~~µL of 5 mg/mL MTT in
308 PBS were added to cells for 3 additional hours at 37 °C. The plates were then centrifuged, the
309 supernatants discarded and the dark blue formazan crystals dissolved using 100 µL of lysis buffer
310 containing 20 % (w/v) sodium dodecyl sulfate, 40 % N,N-dimethylformamide (pH 4.7 in 80 % acetic
311 acid). The plates were then read on Synergy HT microplate reader at a test wavelength of 550 nm and
312 at a reference wavelength of 650 nm.

313

314 **2.7. Microbiological assays**

315

316 ***2.7.1. Determination of vancomycin antimicrobial activity against MRSA***

317 Vm solutions were freshly prepared for each experiment. Determination of the minimum inhibitory
318 concentration (MIC) of vancomycin was carried by the microdilution broth method according to the
319 latest Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). Interpretation of the
320 results was performed as outlined in the above mentioned CLSI guidelines (CLSI 2012).

321

322 ***2.7.2. In vitro antibacterial efficiency of VmLNBS against MRSA.***

323 MRSA, isolated from human ulcerated wounds (Infermi Hospital, Biella, Italy), was cultured over
324 night at 37°C in TSB. After incubation, bacteria were re-suspended in 100 mL of TSB, harvested by 10

min centrifugation at 4,000 rpm, diluted in TSB to 10^4 colony-forming-unit (CFU)/mL, as confirmed by colony counts on TSA, and then incubated in TSB with VmLNBS, loaded with Vm at different concentrations (1, 0.1, 0.01, and 0.004 mg/mL), in sterile sampling tubes for 2, 3, 4, 6, and 24 hours at 37°C. Controls represented by either bacteria incubated in TSB, bacteria incubated with blank NBs or bacteria incubated in the presence of free Vm at different concentrations (1, 0.1, 0.01 and 0.004 mg/mL), were also performed. At each incubation time, serial 10-fold dilutions in saline solution (0.9% NaCl) were prepared from each sample, and 100 ~~micro~~L of each dilution were spread on TSA, so that the number of CFU/mL could be determined.

2.7.3. Imaging with confocal laser scanning microscopy

~~MRSA bacteria *S. aureus* strain~~ ~~were~~~~was~~ grown in TSB at 37°C in agitation until reaching the concentration of 1×10^9 CFU/mL. Then, 1 mL aliquot of bacteria was pelleted (3000g x 10 min at 4°C), resuspended in PBS 1x and incubated with 6-coumarin-labeled VmLNBS, 6-coumarin-labeled NBs, or FITC-labeled Vm at a dilution of 1:11, as for previous experiments performed on eukaryotic cells. Each sample was placed on orbital shaker (160 rpm) in the dark at 37°C for 2h and 4h. After incubation, one drop from each suspension was streaked on poly-L-lysine-coated microscope slides and allowed to dry. Then, bacteria were stained with iodide propidium (PI) in PBS 1X and again allowed to dry. Fluorescence images were taken with an Olympus IX70 inverted laser scanning confocal microscope, and captured using FluoView 200 software.

2.8. Statistical analysis

At least three independent experiments, each one in duplicate or triplicate, were performed for every investigational study. Numerical data are shown as means \pm SEM for inferential results or as means \pm SD for descriptive results (see Cumming et al., 2007 for an exhaustive review). Imaging data are shown

349 as representative pictures. All data were analyzed by a one-way Analysis of Variance (ANOVA)
350 followed by Tukey's post-hoc test (software: SPSS 16.0 for Windows, SPSS Inc., Chicago, IL). $P < 0.05$
351 were considered significant.

352

3. Results

3.1. Characterization of VmLNB and control (blank NB and Vm) formulations

Before NB production, the interaction between dextran sulfate and Vm was firstly investigated to optimize Vm/dextran sulfate ratio. Results indicated that Vm was complexed at 99% by dextran sulfate solution until the concentration of 0.5 mg/mL (data not shown). The Vm/dextran sulfate ratio was calculated corresponding to 2:1 (w/w). Based on this preliminary information, NBs were prepared according to the protocol described in the Materials and Methods section. After manufacturing, VmLNB and blank NB formulations (with or without 6-coumarin in the inner core) were characterized physico-chemically. Results are shown in **Figure 2** and **Tables 1-2**. Both VmLNBs and NBs displayed spherical shapes with a core-shell structure by TEM analyses. All sizes were in the nanometer range, with all formulations displaying around 300 nm as a value for average diameters. All polydispersity indexes were included between 0.22 and 0.25. Zeta potentials ranged from -34 mV (NBs) to -29 mV (VmLNBs). ~~The loading of Vm in the NB structure did not significantly affect the viscosity of the formulations. NB viscosity (1.12 cP) was slightly increased upon binding with Vm (1.25 cP for VmLNBs).~~ NBs were able to load Vm with an encapsulation efficiency of 86% and loading capacity of 29%.

3.2. Stability of VmLNB and control (blank NB and Vm) formulations

NB and VmLNB formulations proved to be physically stable over time, as confirmed by long-term checking of the parameters assessed in the previous paragraph. Indeed, the obtained values did not remarkably change up to six months after the manufacturing of the formulations stored at 4 °C (data not shown). Furthermore, the chemical stability of the drug was comparatively checked between free Vm solution and VmLNB aqueous suspension either over time (up to 14 days) or at different

temperatures (25°C and 37°C). As shown in **Figure 3**, ~~the drug Vm always~~ resulted much more stable ~~from a chemical point of view~~ when properly incorporated in the nanocarriers (VmLNBs) than ~~as such~~ ~~in solution alone~~.

3.3. Human biocompatibility

The potential toxicity of Vm solution and VmLNB suspension on human skin cells was assessed by testing *in vitro* cultured HaCaT keratinocytes. Cells were incubated for 24 h alone, with 10% v/v Vm solution, or with VmLNB nanosuspensions in normoxic conditions (20% O₂). Thereafter, cytotoxicity was analyzed by LDH assay, and cell viability by MTT assay. As shown in **Figure 4**, neither Vm nor VmLNBs did show significant toxic effects and HaCaT cell viability was not significantly affected by either formulation.

3.4. *In vitro* drug release from VmLNBs

In vitro drug release from VmLNB nanosuspension and free Vm solution were comparatively evaluated over time. As shown in **Figure 5** (time course studies up to 6 h) and **Table 23** (end-point data up to 24 h), 1 mg/mL Vm release from VmLNBs was slow and prolonged over time, compared to free drug solution diffusion. No initial burst effect was observed indicating Vm incorporation in NB shell. Further information on additional incubation times and drug concentrations for VmLNBs is available in Supplementary Materials (**Table S34**). Vm/VmLNB drug release ratios at different times (2, 3, 4, 6, and 24 h) were also calculated (see **Table 23**), in order to allow normalization of the results from treatment with VmLNBs in the microbiological experiments described in the following paragraph.

3.5. *In vitro* antimicrobial activity of VmLNBs

401 According to preliminary microbiological analyses performed on the MRSA strain employed in the
402 present experiments, 0.004 mg/mL resulted as the MIC value for Vm. Therefore, decreasing Vm
403 concentrations from 1 mg/mL (used for the studies described in the previous paragraphs) to 0.004
404 mg/mL (MIC value) were employed in a series of experiments aimed at comparatively evaluating Vm
405 (either free or carried by VmLNBs) antibacterial effectiveness against MRSA. Bacteria were incubated
406 at different times (2, 3, 4, 6, and 24 h) either alone (ctr) or with free Vm, VmLNBs, or blank NBs. The
407 initial drug concentrations (1; 0.1; 0.01; and 0.004 mg/mL) loaded on VmLNBs were the same as those
408 solved in free Vm solution. However, as emerged in the previous paragraph, drug release from
409 VmLNBs is significantly slower than free Vm solution diffusion. For this reason, before proceeding
410 with the analysis of the results, all values on bacterial growth referring to Vm- and VmLNB-treated
411 samples were normalized upon time-dependent Vm/VmLNB drug release ratios shown in **Table 23**.
412 Normalized results are shown in **Figure 6**, whereas raw data are available in Supplementary Materials
413 (**Figure S24**). 1 mg/mL Vm effectively inhibited bacterial growth at all times, independently from
414 being free or carried by the nanocarrier. Lower drug concentrations of free Vm solution were effective
415 against MRSA only after longer times of incubation (at least 3 h for 0.1 mg/mL and 0.01 mg/mL Vm;
416 and at least 4 h for 0.004 mg/mL Vm). Interestingly, Vm antibacterial efficacy was significantly
417 improved when the drug was carried by VmLNBs. Indeed, VmLNB-dependent inhibition of bacterial
418 growth was significantly enhanced compared to free Vm solution, at all drug concentrations.
419 Additionally, compared to free Vm solution, VmLNB antibacterial effects appeared earlier, as they
420 were already evident after 2 h of incubation (the first time-point of the observational period) at all Vm
421 concentrations. Blank NBs did not show any antibacterial activity.

422 Further analysis by confocal microscopy (**Figure 7**) displayed that MRSA avidly internalized free
423 fluorescent Vm already after 2 h of incubation, but not fluorescent VmLNBs. Fluorescent Vm-free NBs
424 did adhere to the bacterial cell wall without being internalized.

425 3.6. US-triggered drug permeation

426 The ability of US to promote Vm permeation through the skin was assayed by employing a purposely
427 modified Franz cell constituted by a donor and a recipient chamber separated by a pig skin layer (see
428 **Figure 8A** for a schematic representation of the apparatus). As shown in **Figure 8B**, the administration
429 of US ($t = 10$ min; $f = 2.5$ MHz; $P = 5$ W) strongly induced VmLNBs to deliver the antibiotic drug
430 from the donor chamber throughout the pig skin membrane into the recipient chamber up to 6 h.
431 Furthermore, drug accumulated in the skin after US treatment reached $158 \mu\text{g}/\text{cm}^2$ after 6 hours.

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4. Discussion

Vm currently represents the main stay against MRSA infections ([Koyama et al., 2013](#); ~~Kullar et al., 2016~~ et al., 2016). However, Vm administration raises several issues that urgently need to be faced, including its marked instability, low oral bioavailability, complex concentration-time profile, low tissue penetration (ranging from 10% in diabetic to 30% in normal skin and soft tissues), and several adverse effects (Mawhinney et al., 1992; Raverdy et al., 2013; Vandecasteele et al., 2012; Vidal et al., 1992; Giandalia et al., 2001). In the attempt to counteract these drawbacks, thus improving the effectiveness of Vm delivery, some novel nanocarriers have been developed: i) Vm coupling to chitosan as an ocular drug delivery vehicle for topical use in rabbit eyes has appeared more effective than carrier-free Vm (Khangtragool et al., 2011); ii) PEGylated liposomal Vm enhanced the effective treatment of MRSA pneumonia and simultaneously reduced the nephrotoxicity risk compared with conventional and non-PEGylated Vm formulations (Muppidi et al., 2011); iii) Vm-loaded liposomes, stabilized with chitosan modified gold nanoparticles bounded to their surface, have proven effective in inhibiting the bacterial growth (Pornpattananangkul et al., 2011); and iv) Vm-containing trehalose and hydroxyethylcellulose spherical matrices have been developed as new delivery systems suitable for topical applications on extensive and purulent wounds (Giandalia et al., 2001). Recently, Vm-loaded polymersomes were developed from a novel pegylated oleic acid polymer for sustained antibiotic delivery (Omolo et al., 2017). Overall, these works represent the proof-of-principle for the feasibility of choice of nanocarriers, as alternative drug delivery systems to obtain the desired drug release rates and bioavailability (Kalhapure et al., 2015). However, the effectiveness of those nanocarriers was seriously undermined by their poor ability to cross the *stratum corneum*, a skin barrier displaying low permeability unless proper exogenous physical stimuli are provided (Azagury et al., 2014; Park et al., 2012).

For these reasons, the present study aimed at developing Vm nanocarriers as a new platform to be effectively and safely employed for Vm topical administration to treat wound infections. To this purpose, NBs with core-shell nanostructures were identified as first choice carriers due to their known benefits in association with drug delivery, including small size, stability, suitability for drug loading, responsiveness to external stimuli such as US, and controlled drug release abilities (Marano et al., 2016; Cavalli et al., 2009a; Cavalli et al., 2009b; Cavalli et al., 2016). In this study, dextran sulfate was chosen as main constituent of the polysaccharidic shell as a consequence of the large amount of data from the literature supporting dextran biocompatibility (Bos et al., 2005; De Groot et al., 2001). Encouragingly, dextran-based hydrogels have already been employed as matrices in tissue engineering, without showing signs of inflammation *in vivo* (Möller et al., 2007), and recent toxicological studies have shown that dextran, as well as the products from its mechano-chemical processing, can be classified as class 4 (low-toxicity) substances (Dushkin et al., 2013). Moreover, dextran sulfate presents a negative charge that can electrostatically interact with the positive charged Vm. On the other hand, PFP was employed as principal constituent of the inner core, since it is the most widely used fluorocarbon in oxygenating emulsions and NB formulations (Cabral and Intaglietta, 2013, Castro and Briceno, 2010). In order to load Vm, dextran sulfate-shelled/PFP-cored NBs were then functionalized by exploiting the electrostatic interactions occurring between the negatively charged sulfate groups of the shell and the protonated amino groups of the drug. The obtained VmLNBS displayed a spherical shape and a well-defined core-shell structure with a polymeric shell thickness of about 40 nm, average diameters of 300 nm, viscosity of 1.25 cP, and negatively charged surfaces. Of note, the observed decrease of zeta potential values of ~ 15 % for VmLNBS (around -29 mV) with respect to blank NBs (around -34 mV) confirmed the occurrence of electrostatic interactions between positive amino groups of the drug and negative sulfate groups of the polymer, leading to a partial

charge neutralization of the bubble surface and allowing a good Vm encapsulation efficiency (86%) and loading capacity (29%) in the NB systems.

In addition, it should be noticed that since the zeta potential measures charge repulsion or attraction between particles, it represents a fundamental parameter to avoid nanoparticle aggregation, with zeta potentials lower than -25 mV or larger than +25 mV being generally required for physical stability of colloid systems (Shah and Eldridge, 2014). The stability of VmLNB formulations was further confirmed by long-term checking of their size, surface charge, and viscosity values, which did not show any significant changes up to six months after manufacturing, stored at 4 °C. On the other hand, drug stability was comparatively checked between free Vm solution and VmLNB suspension either over time (up to 14 days) or at different temperatures (25°C and 37°C), revealing an increased stability for Vm when properly encapsulated in the nanocarriers. This appears as an undoubtedly advantageous feature for VmLNB formulations, since they might prove useful to overcome the reported instability of Vm in aqueous solutions at body temperature (Mawhinney et al., 1992, Raverdy ~~V~~-et al., 2013). Interestingly, VmLNBs displayed a slow and prolonged drug release kinetics compared to Vm aqueous solution, with only 16% of the drug being released from VmLNBs after 6 h. These data support the hypothesis that VmLNBs may be employed as an effective drug reservoir until reaching the target site, where the antibiotic would be released upon sonication at an appropriate moment only. The features of VmLNBs might be exploited for the design of innovative wound dressing following their inclusion in polymeric base. Indeed, NBs can be dispersed in polymer gel without changing physico-chemical characteristics, as previously showed (Prato et al., 2015). Another intriguing feature of VmLNBs relies on the reported evidence that surface charges play a pivotal role in making a nanoparticle suitable for topical treatment, since they enhance its interaction with the skin and improve its therapeutic effect on inflamed cutaneous tissues, either without (Abdel-Mottaleb et al., 2012) or with concomitant US treatment (Lopez et al., 2011). Although cationic nanoparticles are generally preferred for topical

506 treatment due to the anionic nature of the skin (Wu et al., 2010), some authors have shown that anionic
507 nanoparticles can be more effective (Lee et al., 2013) and less toxic (Ryman-Rasmussen et al., 2007)
508 than the cationic ones. These latter data appear consistent with our results through investigation by
509 biochemical assays to assess VmLNB biocompatibility with human skin tissues. Indeed, VmLNBs did
510 not induce any *in vitro* cytotoxic effects on HaCaT keratinocytes, a skin cell line that was originally
511 immortalized from a 62-year old donor (Boukamp et al., 1988). This peculiar information strengthens
512 remarkably the evidence on VmLNB safety for future topical applications.

513 VmLNB and carrier-free Vm antimicrobial activity against MRSA were comparatively investigated,
514 also analyzing Vm and NB physical interaction with the bacterial cell wall by confocal microscopy.
515 Interestingly, VmLNBs were more effective in MRSA bacterial growth inhibition than free Vm,
516 promoting enhanced and earlier antibacterial effects, although they were not internalized by bacteria,
517 opposite to free Vm. This behavior appears to be a likely consequence of time-sustained release of Vm
518 from VmLNBs.

519 Notably, an important issue that requires caution while evaluating the feasibility for any topical drug
520 treatment is represented by the considerably low degree of permeability of the skin, the primary
521 defense system for the body. This organ consists of several layers, including the *stratum corneum*, the
522 epidermis, and the dermis. In particular the *stratum corneum* - composed of corneocytes interspersed in
523 a laminate of compressed keratin and intercorneocyte lipid lamellae - is very poorly permeable to
524 foreign molecules and represents the main obstacle to transdermal drug delivery (Naik et al., 2000).
525 However, an ideal antibiotic drug formulation should be efficiently localized in the epidermis/dermis
526 and provide a sustained drug release over time (Prabhu et al., 2012). To allow a drug to penetrate the
527 skin, several approaches have been proposed, including skin patches, ionophoresis, chemical
528 enhancers, and US-triggered sonophoresis (Park et al., 2014).

529 Interestingly, antimicrobial properties have been reported for US, although its effectiveness strongly
530 varies depending on the targeted type of pathogen (fungi vs bacteria; cocci vs bacilli; Gram-positive vs
531 Gram-negative) (Sango et al., 2014). Furthermore, synergistic effects between US and antibiotics have
532 been reported in a series of studies: i) antibiotic treatment coupled with US irradiation resulted in
533 enhanced bactericidal activity against both Gram-positive and Gram-negative bacteria, especially for
534 aminoglycosides (Yu et al., 2012); ii) the combination of Vm and US decreased *S. aureus* viable counts
535 by two orders of magnitude compared to Vm alone (Ayan et al., 2008); and iii) the addition of NB-
536 enhanced US to doxycycline treatment improved the drug effectiveness in eradicating intracellular
537 *Chlamydia trachomatis* (Ikeka-Dantsuji et al., 2011). US-dependent enhancement of antibiotic action
538 on biofilms was named as a 'bioacoustic effect'. Interestingly, Vm transfer through *S. epidermidis*
539 biofilms was shown to be significantly enhanced by US, with bubbles being able to increase the biofilm
540 permeability to Vm (Dong ~~Y~~-et al., 2013).

541 As discussed previously, VmLNBs can be effectively employed as an important reservoir to store the
542 drug until trespassing the *stratum corneum* of the skin and reaching the target site. In order to achieve
543 the latter goal, US was assayed for its ability to induce VmLNBs to trespass an *in vitro* cutaneous layer,
544 thereby releasing Vm throughout the skin. Notably, the skin from the pig ear is widely recognized as a
545 good model for human skin permeability, since it displays human-like histological and physiological
546 properties, including epidermal thickness and composition, dermal structure, lipid content and general
547 morphology (Dick ~~and Scottet-al.~~, 1992). The validity of the porcine model has been established by
548 comparing the permeability of simple marker molecules with the corresponding values across human
549 skin (Herkenne et al., 2006, Sekkat et al., 2002). Therefore, the porcine ear skin represents so far the
550 most accountable *in vitro* model to mimic the human skin in studies on percutaneous penetration
551 (Jacobi et al., 2007). In our experiments, US appeared essential to promote Vm release from VmLNBs
552 throughout the pig skin layers, in line with previous reports on NBs and sonophoresis. On the contrary,

553 the passive transport of free vancomycin hydrochloride was negligible, being a charged and hydrophilic
554 molecule. The amount of Vm accumulated in the skin after US application combined with NBs was
555 greater than MIC value.

556

557 **5. Conclusions**

558 | In the present work, dextran sulfate-shelled and ~~PFP~~perfluoropentane-filled NBs were developed for
559 Vm delivery. VLNBS proved to be effective in MRSA bacterial killing without showing toxic effects
560 on human keratinocytes. The combination of NBs and US enhanced Vm permeation through pig skin
561 and promoted drug skin accumulation. Based on these results, Vm topical administration through
562 proper NB formulations might be a promising strategy for the local treatment of MRSA skin infections.
563 The study represents the proof of concept for the future development of advanced multifunctional
564 therapeutic systems to treat infected wounds.

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832 **Figure legends**

833

834 **Figure 1. Schematic structure of VmLNB formulations.** Vm nanocarriers described in the present
835 work display a core-shell structure. PFP was employed as core fluorocarbon, whereas dextran sulfate
836 was chosen as polysaccharidic shell molecule. Vm was inserted into the outer shell throughout dextran
837 sulfate chains. In selected experiments, VmLNBS were further functionalized by including fluorescent
838 6-coumarin in the inner core.

839

840 **Figure 2. NB and VmLNB morphology.** NBs and VmLNBS were checked for morphology by TEM.
841 Results are shown as representative images from three different preparations. Panel A. NB image by
842 TEM. Panel B. VmLNB image by TEM. [\(see also Figure S1 in Supplementary Materials for additional](#)
843 [images of multiple nanobubbles within the same field\).](#)

844

845 **Figure 3. Stability of Vm and VmLNB formulations.** The stability of Vm solution and VmLNB
846 suspension was monitored up to 14 days either at room temperature (Panel A) or at 37°C (Panel B)
847 through analysis by HPLC. Results are shown as means \pm SD from three different preparations for each
848 formulation. Data were also analyzed for significance by ANOVA. Versus Vm solution: * $p < 0.001$.

849

850 **Figure 4. Biocompatibility of Vm and VmLNBS with human keratinocytes *in vitro*.** HaCaT cells
851 (10^6 cells/2 mL DMEM medium implemented with 10% FCS) were left untreated (ctr) or treated with
852 200 ~~micro~~μL of Vm solution or VmLNB suspension for 24 h in normoxia (20% O₂). Thereafter, Vm
853 and VmLNB cytotoxicity were measured through LDH assay (Panel A), whereas cell viability was
854 measured through MTT assay (Panel B). Results are shown as means \pm SEM from three independent

855 experiments. Data were also evaluated for significance by ANOVA. No significant differences were
856 found among all conditions.

857 **Figure 5. *In vitro* Vm release from Vm and VmLNB formulations.** Vm release from Vm solution
858 and VmLNB suspension was monitored up to 6 h. Results are shown as means \pm SD from three
859 different preparations for each formulation. Data were also analyzed for significance by ANOVA.
860 Versus Vm solution: * $p < 0.001$.

861

862 **Figure 6. Antibacterial activity of Vm and VmLNBs against MRSA.** MRSA were left for 2, 3, 4, 6
863 and 24 hours at 37°C alone (ctr) or incubated with 10% v/v NBs or different concentrations of Vm,
864 either free or loaded on VmLNBs (Panel A: 1 mg/mL; Panel B: 0.1 mg/mL; Panel C: 0.01 mg/mL;
865 Panel D: 0.004 mg/mL). Results are shown as means \pm SEM from three independent experiments. Data
866 on Vm- and VmLNB-treated samples were normalized upon Vm/VmLNB release ratios reported in
867 Table [23](#) (see also in Supplementary Materials: Table [S34](#) for further information on percentages of
868 drug release from VmLNBs at different times/concentrations; and Figure [S24](#) for raw data on VmLNB
869 antibacterial effects). All data were also evaluated for significance by ANOVA. Versus ctr: * $p < 0.02$;
870 versus Vm: ° $p < 0.05$.

871

872 **Figure 7. Drug loading on dextran sulfate-shelled NBs prevents Vm internalization by MRSA.**
873 MRSA were left alone or incubated with 10% v/v 6-coumarin-labeled VLNBS, 6-coumarin-labeled
874 NBs, and FITC-labeled Vm for 2h at 37°C. After staining bacteria with PI, confocal fluorescent images
875 were taken using FITC and TRITC filters. Data are shown as representative images from three
876 independent experiments. Magnification: 100X. Red: PI. Green: 6-coumarin or FITC.

877

878 **Figure 8. US-triggered sonophoresis of VmLNBs through skin membranes.** US ($t = 10$ min; $f = 2.5$
879 MHz; $P = 5$ W) abilities to induce sonophoresis and Vm permeation from VmLNBs were evaluated up
880 to 6 h by using a vertical diffusion Franz cell consisting in two chambers (donor and recipient,
881 respectively) separated by a pig skin layer (see scheme in Panel A). Results are shown in Panel B as
882 means \pm SD from three independent experiments. Data were also evaluated for significance by
883 ANOVA. Versus without US: $p < 0.001$.

884 **Tables and legends**

885

Formulation	Average diameter	Polydispersity	Zeta Potential	<u>Viscosity</u>
	± SD (nm)	index	± SD (mV)	<u>(cP)</u>
NBs	313.4 ± 26.4	0.24 ± 0.02	- 34.5 ± 0.38	<u>1.22</u>
VmLNBs	304.6 ± 14.6	0.22 ± 0.03	- 28.6 ± 1.34	<u>1.25</u>
Fluorescent NBs	312.8 ± 22.7	0.25 ± 0.02	- 34.1 ± 1.22	<u>1.24</u>
Fluorescent VmLNBs	308.9 ± 22.4	0.23 ± 0.01	- 29.5 ± 1.88	<u>1.23</u>

886

887 **Table 1. Physical-chemical characterization of NBs and VmLNBs.** Liquid formulations were
888 characterized for average diameters, polydispersity index, and zeta potential by light scattering. The
889 viscosity (cP) of NB and VmLNB suspensions was determined at 25 °C by using a Ubbelohde capillary
890 viscosimeter. Results are shown as means ± SD from three preparations. See also Figures 1-2 for
891 further detail on NB and VmLNB structure and morphology.

892

Sample solution	Viscosity (cP)
Vm-	0.98
NBs	1.12
VmLNBs	1.25

893

894 ~~**Table 2. Viscosity of Vm solution, NB, and VmLNB suspensions.** The viscosity (cP) of NB and~~
895 ~~VmLNB suspensions as well as free Vm solution was determined at 25 °C by using a Ubbelohde~~
896 ~~capillary viscosimeter. The results are reported in the table.~~

897

Formatted Table

898

time (hours)	% drug release from Vm solution	% drug release from VmLNBs	Vm/VmLNB drug release ratio
2	36.57	5.99	6.11
3	45.97	7.97	5.78
4	57.16	10.27	5.57
6	73.44	14.59	5.03
24	92.34	35.84	2.58

899

900 | **Table 23. *In vitro* drug release from Vm solution and VmLNB suspension.** After incubation for
901 increasing times (first column), the percentages of *in vitro* drug release from Vm solution (second
902 column) and VmLNB suspension (third column) were measured. Then, Vm/VmLNB drug release
903 ratios (fourth column) were calculated for each time considered. All incubation times (2, 3, 4, 6, and
904 24 h) were purposely chosen to further normalize the results from the experiments with MRSA (see
905 Figure 6). Results are shown as mean values from three different preparations for each formulation.

Figure 1

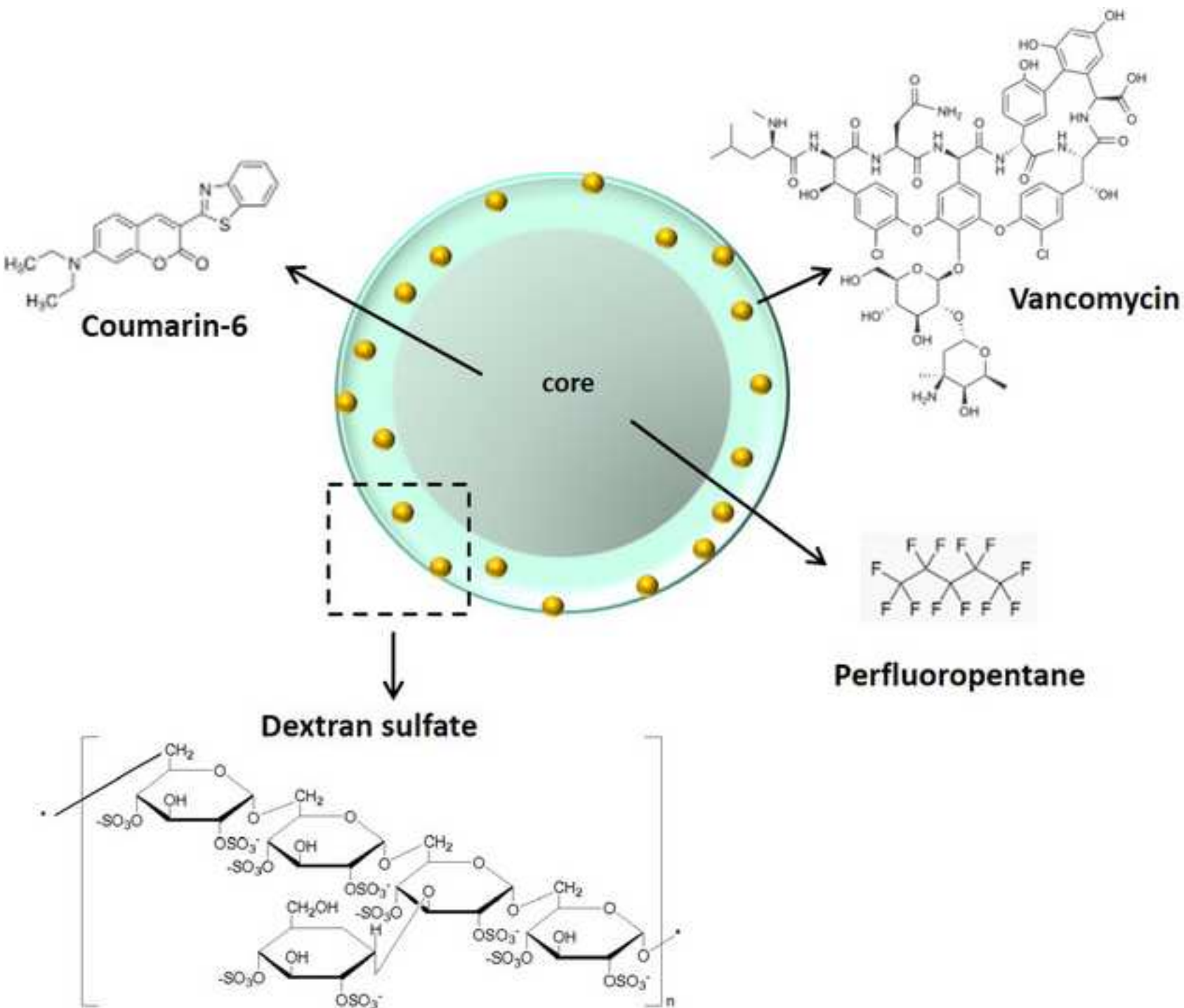


Figure 2

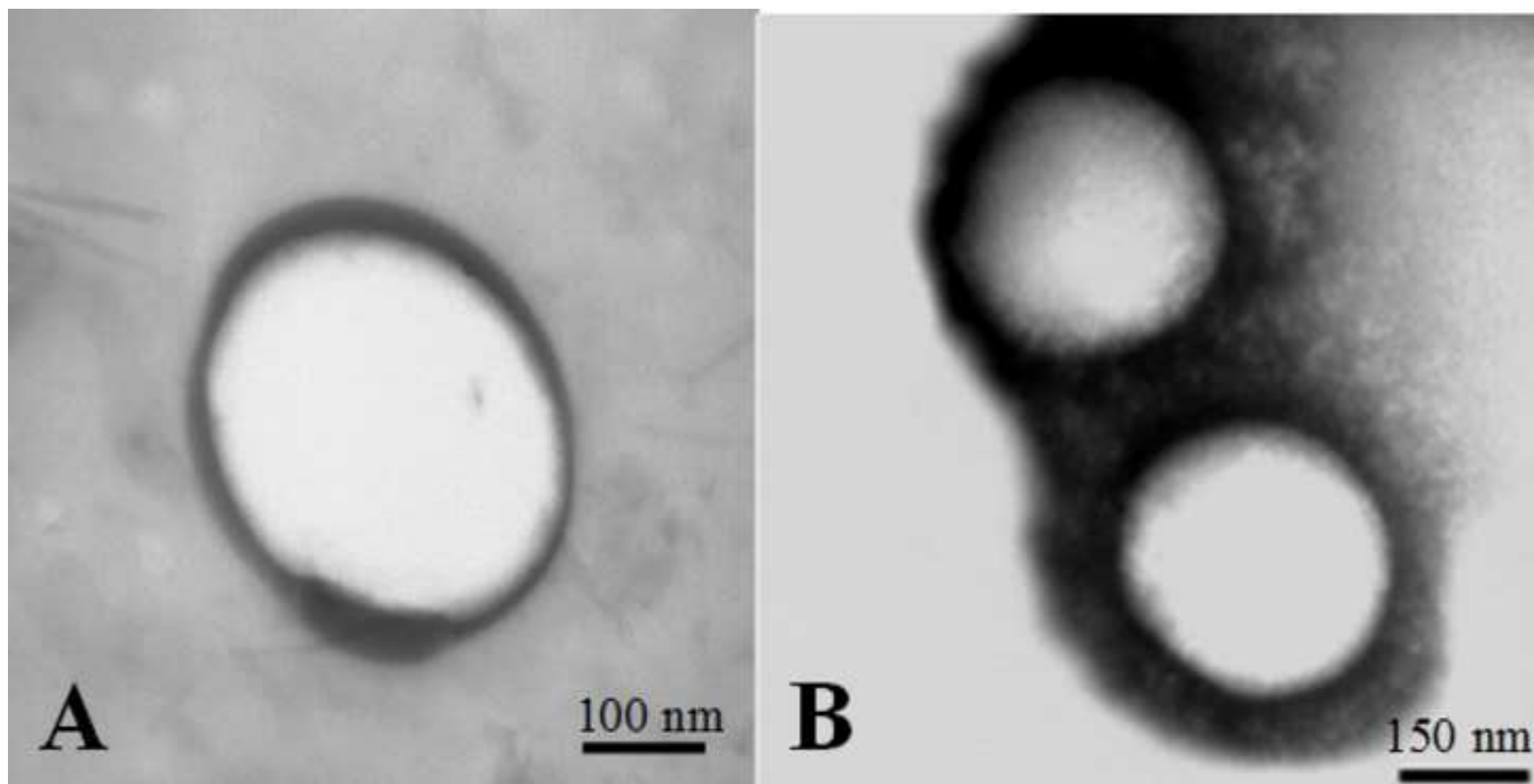


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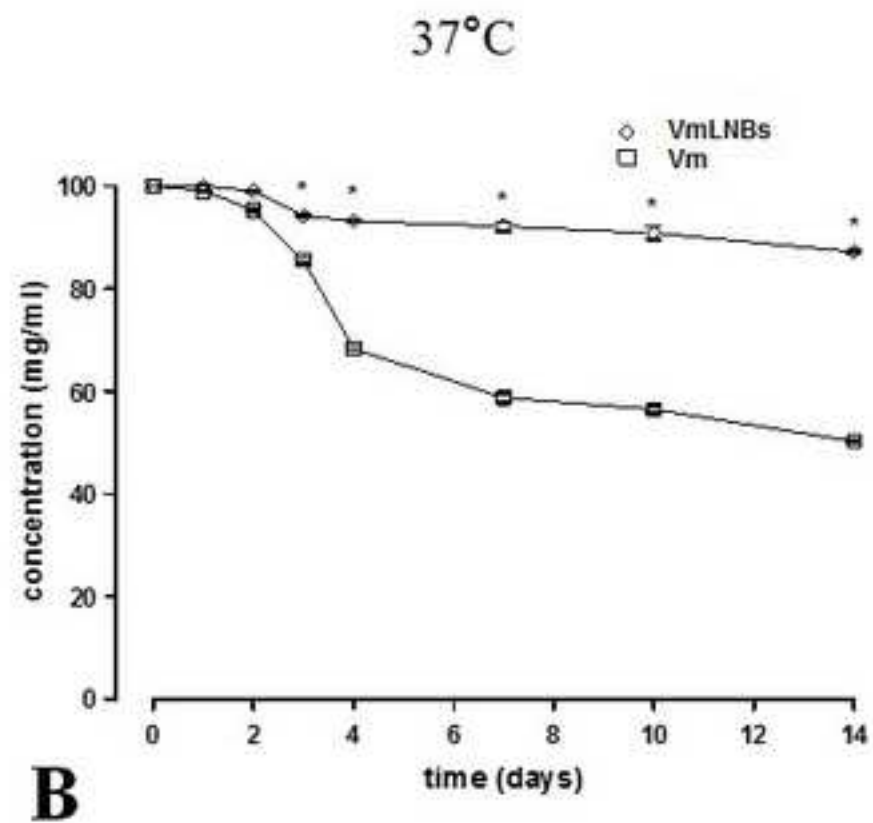
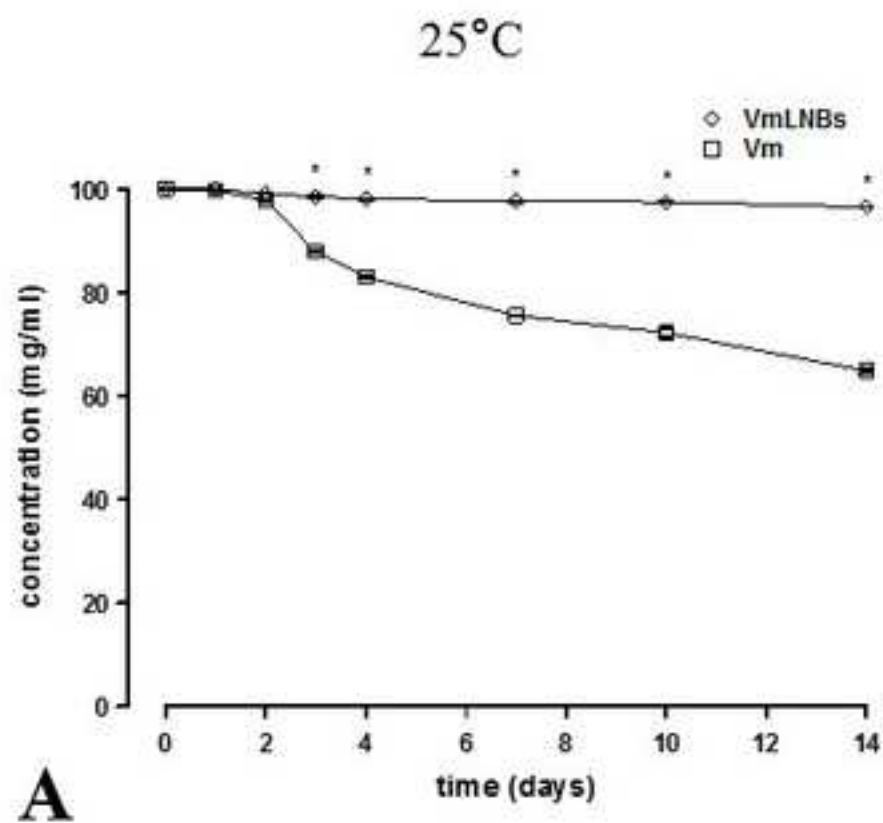


Figure 4

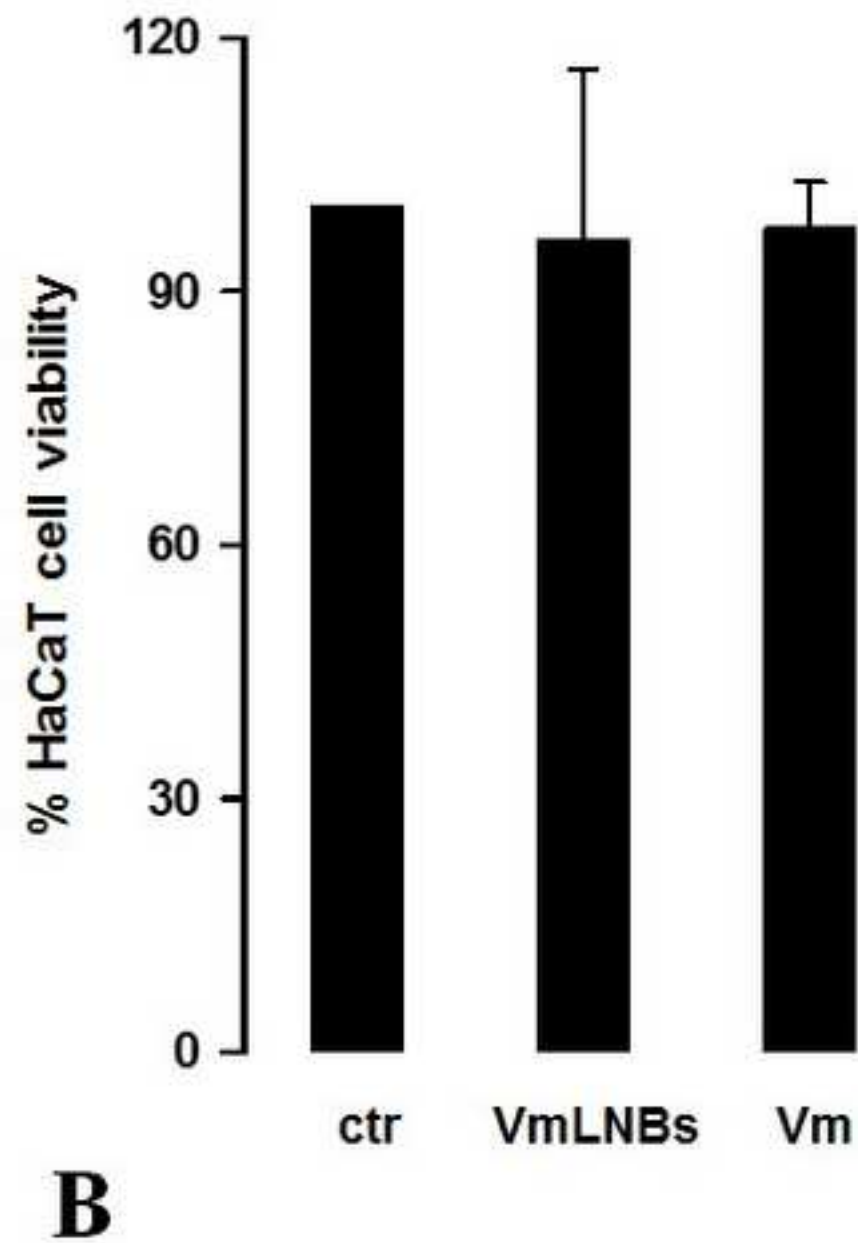
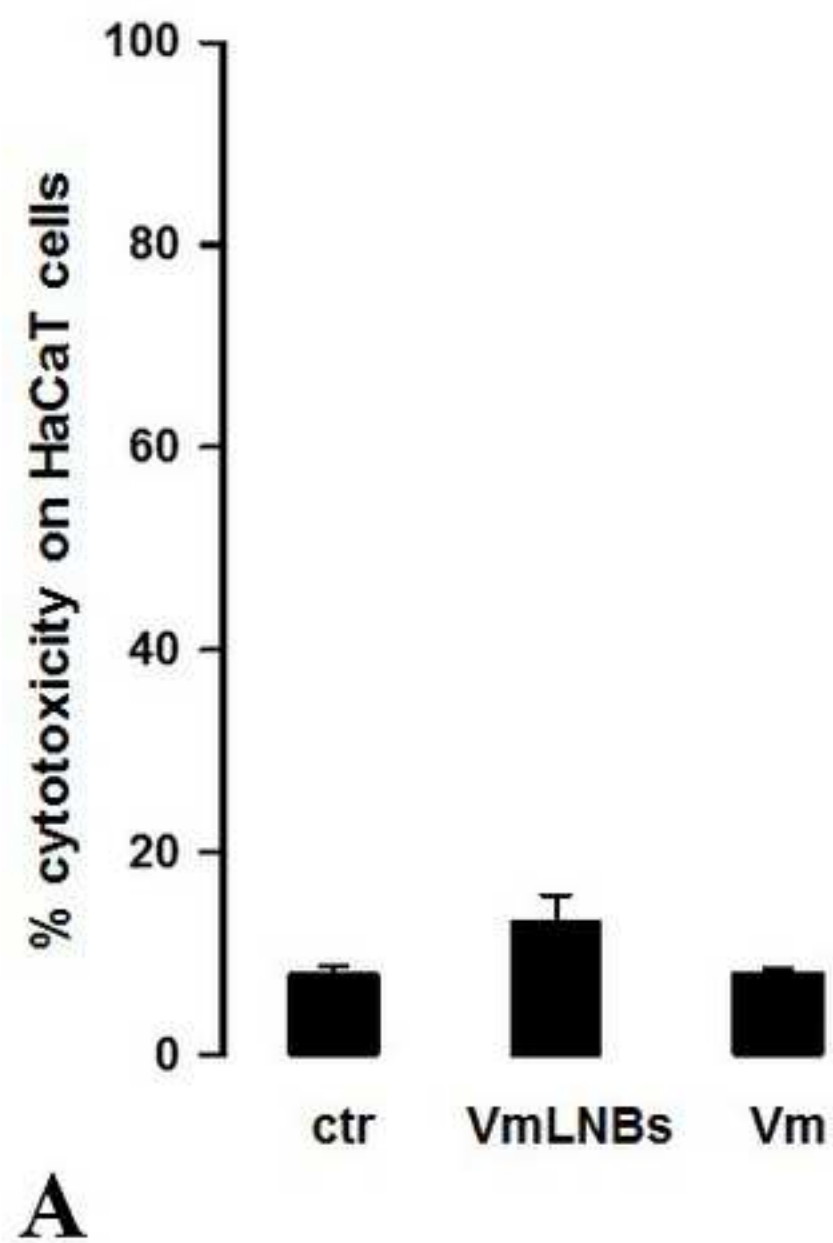


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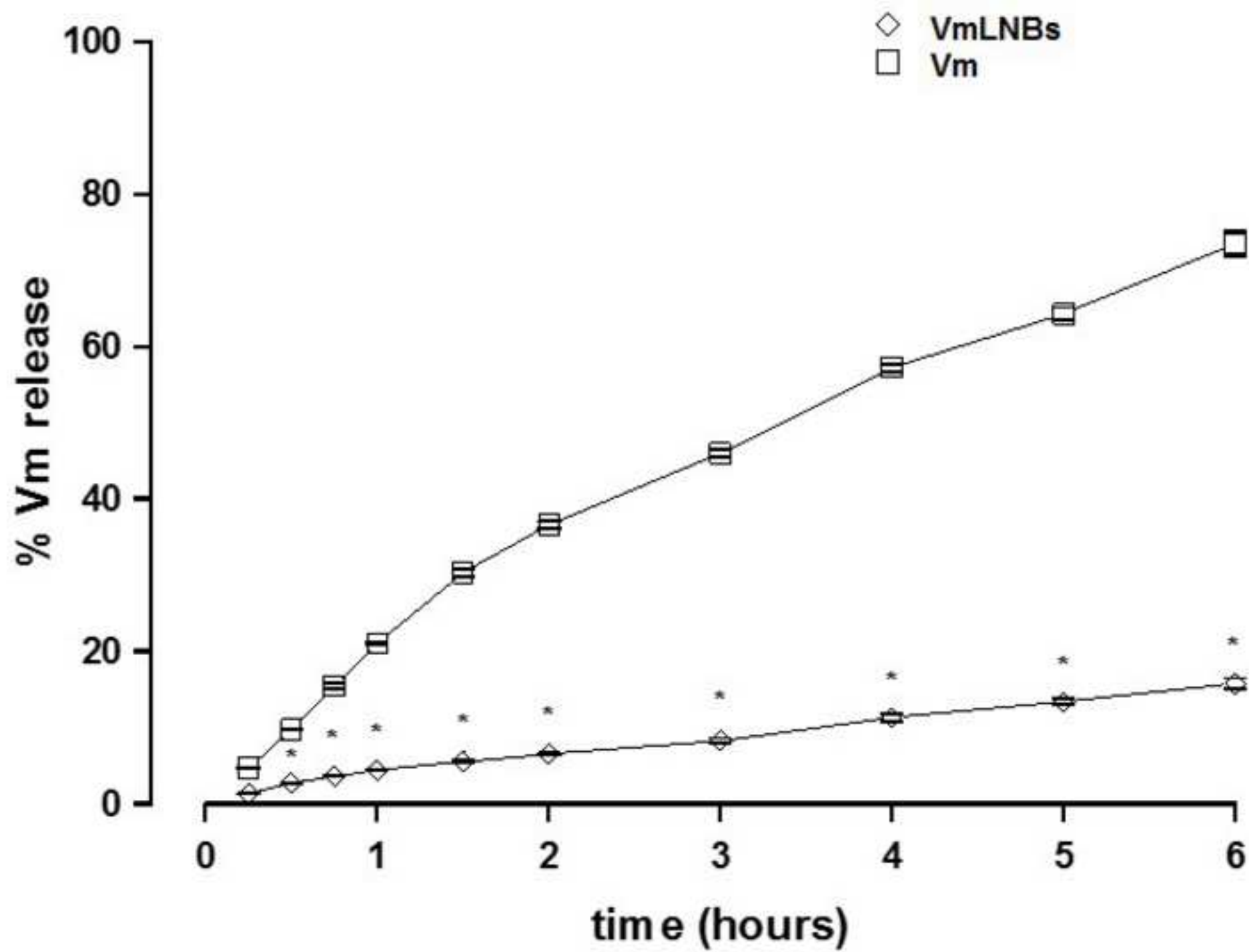


Figure 6

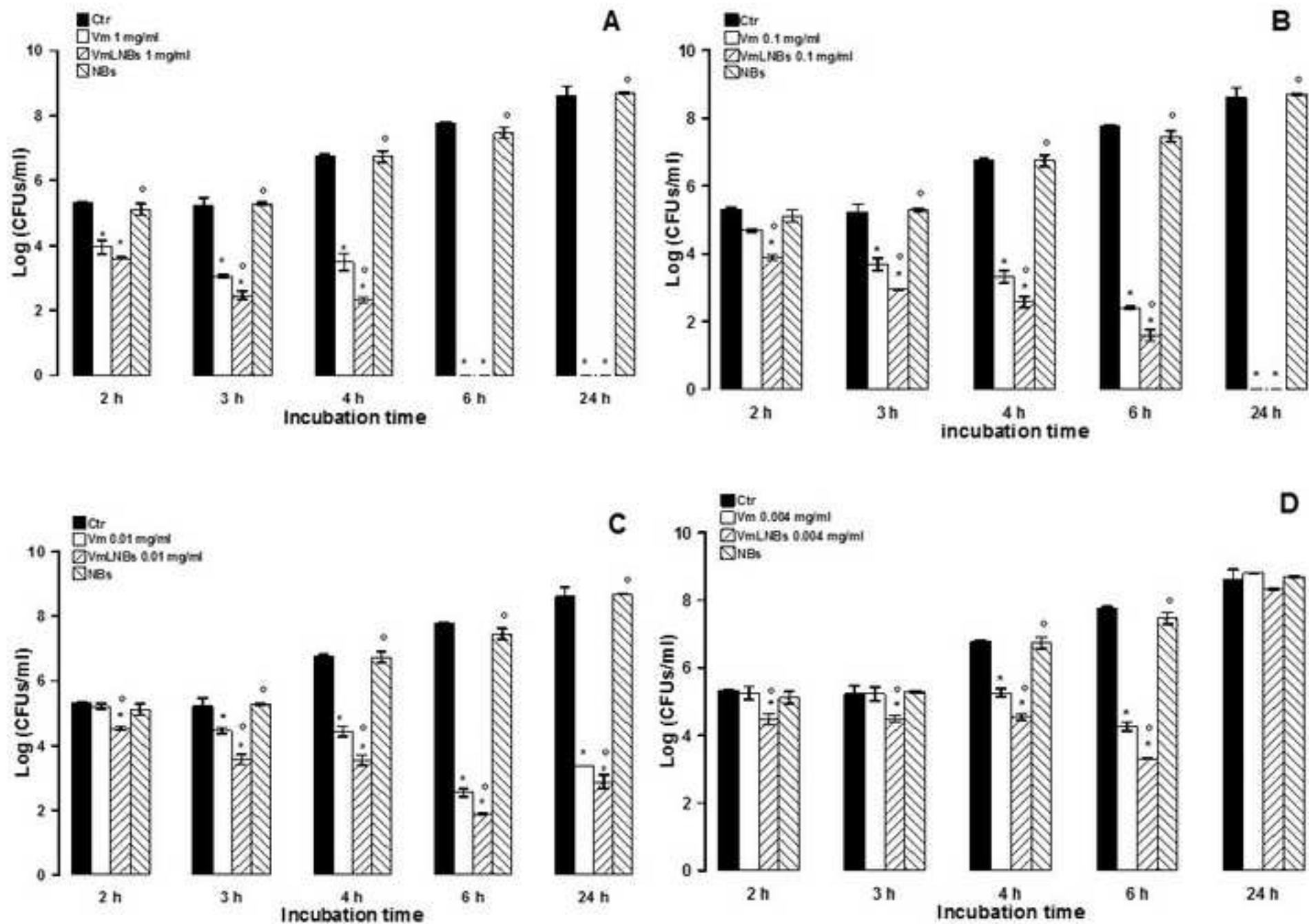


Figure 7

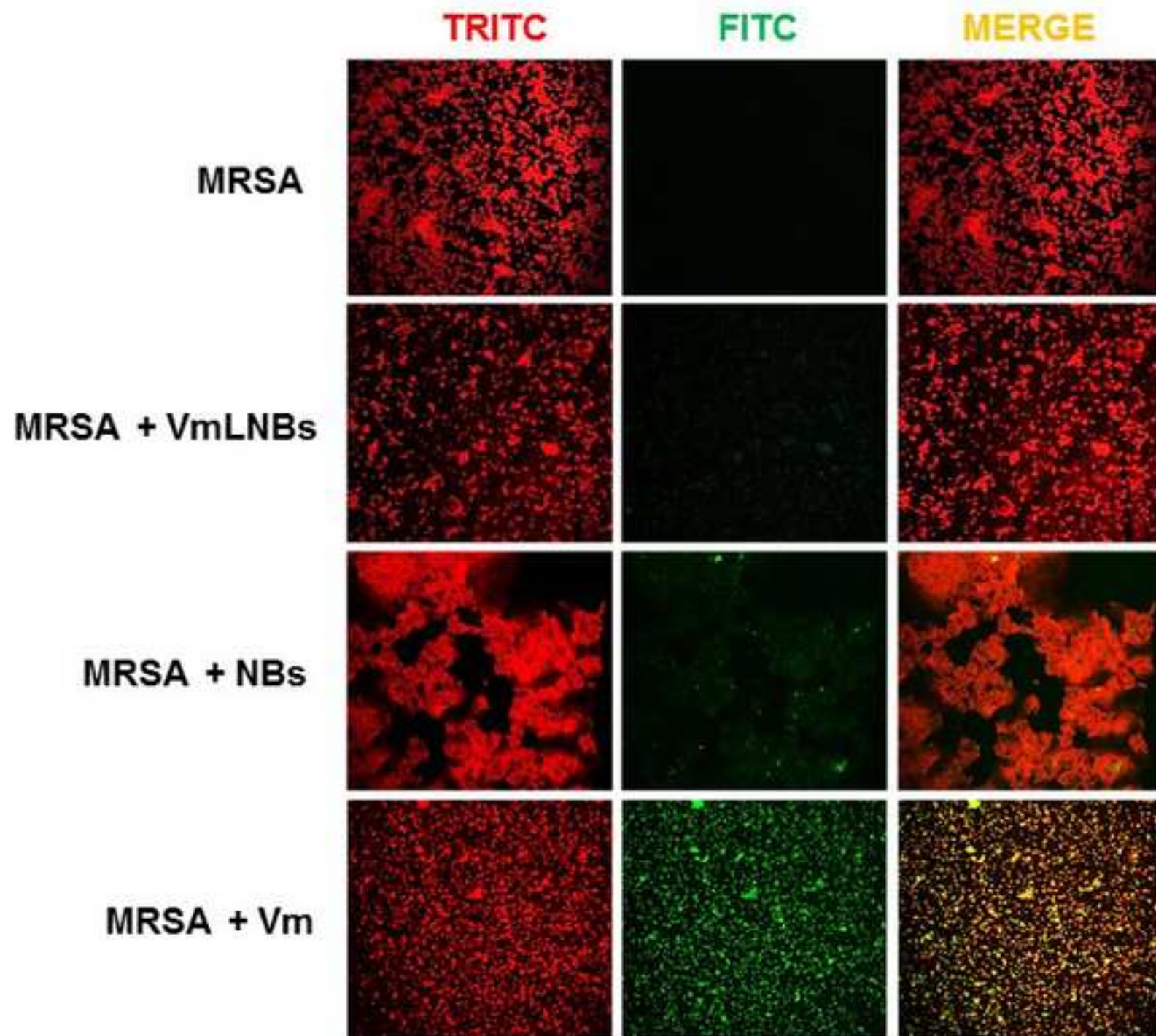
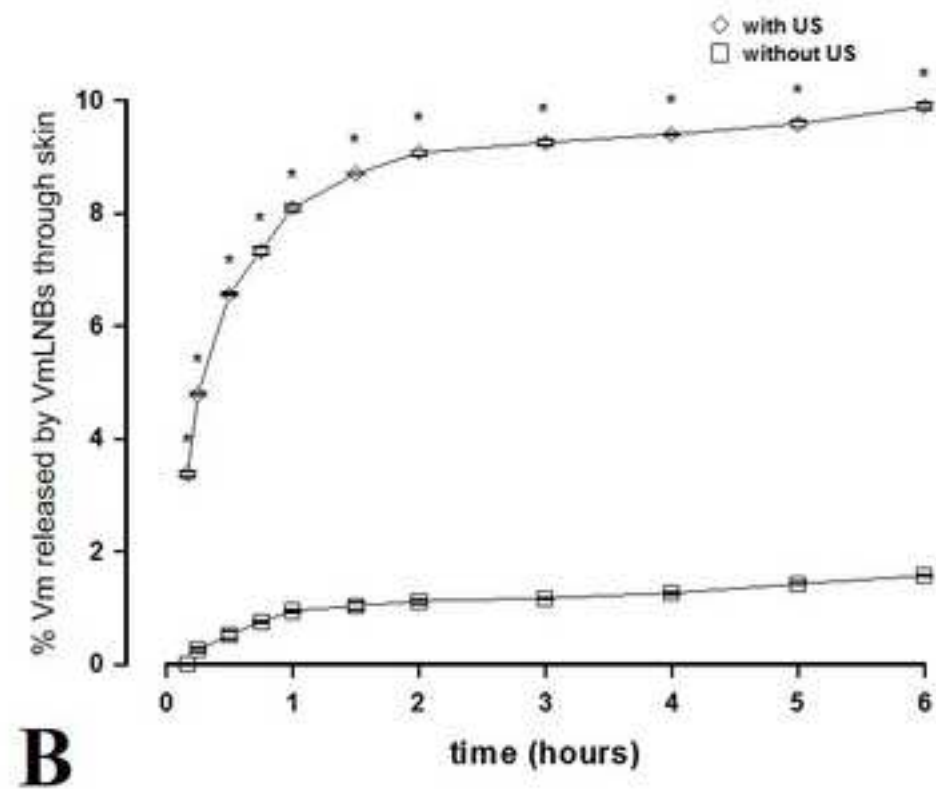
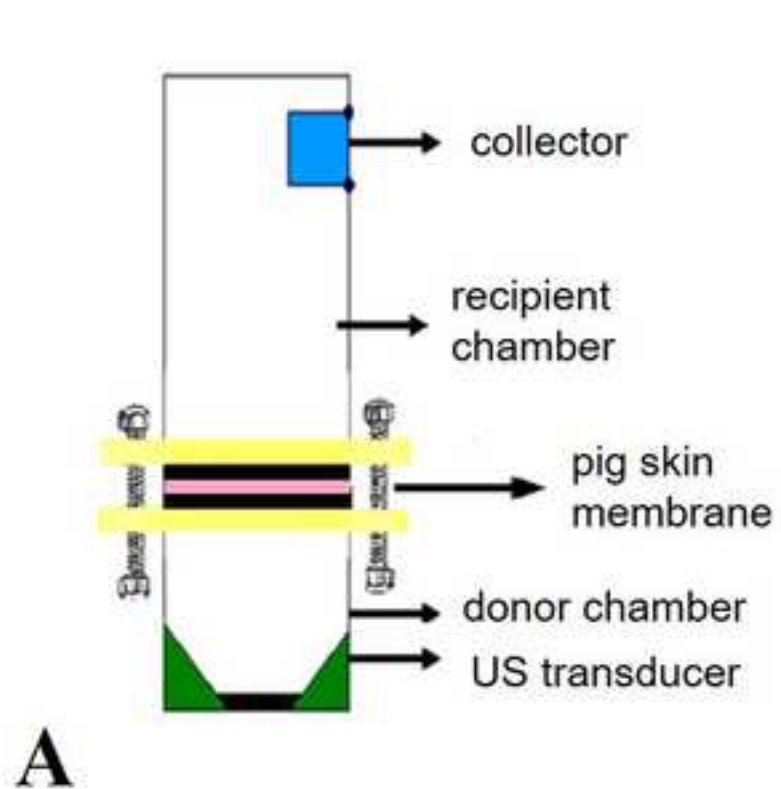


Figure 8



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